

Playing dirty with the China card

Science, the US weapons laboratories, and a hard-working immigrant group are all losers in Washington's game of pinning blame for an alleged security leak at Los Alamos.

Spies stories always thrive on grotesque stereotypes, and the allegation that US nuclear secrets were leaked to China by a Taiwanese-born scientist at the Los Alamos National Laboratory is no exception. Of course national security must be protected. But some press reports in the wake of unproven allegations have presented an unsavoury and unsubstantiated picture of the role of Chinese-American scientists in the United States. In this climate, it is imperative that the US scientific leadership spells out the extent to which US science is indebted to this group, and to the principle of free global exchange of information between scientists.

The People's Republic of China is today the largest source of scientific immigration to the United States, by a considerable margin. According to figures compiled last year by the National Science Foundation (NSF), China supplies well over one-third of the 8,000 foreigners who receive doctorates in the United States each year. Chiefly because of legislation which Congress passed in 1992, as a form of retaliation against the Chinese government for its treatment of student protesters in Tiananmen Square, the Chinese students are automatically entitled to stay in the United States. According to the NSF, 90% of them intend to do so.

The next largest group of immigrants comes from India — another country whose scientists some branches of the United States government are inclined to view with suspicion. This generation of young Asians represents a critical component of the future of US science. Most of these young scientists will become citizens of the United States. They will be just as committed to America as the smaller groups of European scientists who have flowed into the United States, in fits and starts, for most of the century.

The Asian influx comes at a time when, according to studies too numerous to mention, qualified American-born students are not prepared to enter doctoral studies in numbers sufficient to satisfy the demands of the US research universities. It is the overwhelming view of the academic and industrial experts that these immigrant scientists are filling slots that would otherwise go unfilled, and that their

presence in the United States is of fundamental importance to the growth and development of science in the United States.

Ironically, in light of the reaction to the Los Alamos saga (see page 447), a US Commission on Maintaining US Nuclear Weapons Expertise has just reported that the weapons laboratories are already having trouble recruiting scientific staff. Like everyone else, these laboratories are going to have to hire where the talent is. But they are under increasing pressure from the Congress to subject recruits, as well as visitors, to ever more vigilant security checks. But the introduction of polygraph tests and other counter-intelligence procedures at the laboratories can only complicate the already considerable challenge they face in attracting staff. Any hint of a clamp-down on recruits with foreign backgrounds will further isolate the laboratories.

The greatest burden of any such clamp-down would fall, of course, on the Chinese-American scientists themselves. It is incumbent on the American Physical Society and other scientific societies to tell the public the truth about this matter. Chinese-American scientists play a large and increasing role in maintaining US science. These scientists are here at the express invitation of the US Congress; their presence is China's loss and America's gain.

More support should be forthcoming from national leaders in support of Chinese-Americans' role in science and society. President Bill Clinton is constrained, unfortunately, by the circumstances in which the Los Alamos leak — which actually occurred in 1988 — has sprung to prominence in Washington. Clinton and his Democratic Party have long been accused of taking money, through intermediaries, from the communist government of the People's Republic of China. The communists almost certainly did make such donations — after they realized that Taiwan had bought access to the Republican Party. Now the Republicans seek to exploit the Los Alamos leak to portray Clinton as soft on China. This is a puerile Washington mudfight which nobody will win: the losers are likely to include the weapons laboratories, a hard-working and immensely talented immigrant group, and US science. □

Millennial mindsets

Parody can be a powerful weapon — provided it is recognized as such.

As we approach the twenty-first century, recent events in Lawrence, Kansas, give cause for pause. When creationists sought to have evolution taught as theory, not fact, in the Lawrence public schools, a group of scientists and educators responded by forming a group called FLAT (Families for Learning Accurate Theories), challenging the "Round Earth Theory" while arguing that our planet may be a tetrahedron (see page 453).

Incredibly, FLAT's statements at a press conference were reported by some media with the same objectivity as an announcement of the latest rise in wheat sales. The fact that the parodistic pronouncements

of FLAT could be so treated provides a disturbing window on the world where creationists have been able to successfully sow their revisionist concepts in the minds of America's heartland. What spurred this burst of creationist aggravation was a mother's objection to a kindergarten lesson where a plastic dinosaur model was appropriately placed on a chronological chart of the Earth's development.

It must indeed be challenging to teach the products of the last few centuries' thought in an intellectual climate sometimes reminiscent of the Middle Ages. Satire will, alas, continue to be well merited for the foreseeable future. □



US societies fear clamp-down on visits by foreign scientists

[WASHINGTON] Scientific societies in the United States are increasingly concerned that the US government has reversed its previous support of free communication between scientists. They claim there is now a policy of isolating scientists who come from countries to which it wishes to deny access to a range of new technologies.

The societies say, for example, that the State Department is dragging its feet in granting visitors' visas for foreign scientists wishing to attend conferences in the United States. At last month's meeting of the International Union of Pure and Applied Physicists in Atlanta, Georgia, for example, prominent physicists from China and Russia were excluded. Furthermore, the leader of the Indian delegation was only admitted after the personal intervention of Bruce Alberts, president of the National Academy of Sciences.

Irving Lerch, head of international affairs at the American Physical Society (APS), says the United States has reversed course since the last such conference was held here in 1987. Then, visits from Russian and Cuban scientists were welcomed. "Now the situation is quite different," he says. For the State Department, Lerch says, "the most important aspect is to prevent technology transfer".

The State Department has long been try-



Off limits: the Los Alamos National Laboratory in New Mexico, site of the recent controversy over the alleged theft of critical atomic secrets. Scientific societies in the United States see this as having accelerated a trend against allowing foreign scientists to work in 'sensitive' areas of research. Chinese-American scientists feel particularly vulnerable.

ing to curtail visits of scientists from so-called 'rogue states' such as Iraq and Libya. But it was in the aftermath of India and Pakistan's nuclear tests last spring that US scientific societies began to complain about its policies. They now fear that the clamp-down has been lent new impetus by recent allegations that a Taiwanese-born scientist was involved in a security breach at the Los Alamos National Laboratory (see *Nature* 398, 96 & 276; 1999).

The department also appears to be imple-

menting its infrequently exercised right to vet immigrant visa applications from scientists in a wide range of disciplines. Last month, for example, Jianjun Hu, a Chinese materials scientist who has secured a position at Northwestern University, Illinois, had his visa application delayed in circumstances Lerch describes as "clearly fallout from the case at Los Alamos".

The consulate to which Hu applied for a visa referred the application to the State Department on the basis that he would be working on "materials technology". Northwestern expects Hu's visa to be approved within the next few days, a university spokeswoman says. Maria Rudensky, a spokeswoman for the State Department, said this was a well-established procedure. "Most scientists on our 'technology alert list' have to come through this," she said.

The list covers some 20 disciplines, ranging from nuclear and missile technology to advanced computing, microelectronics and 'biotechnology engineering'. It applies to scientists from an equally long list of countries, including Russia, China, Pakistan and South Africa — but not India. Rudensky said the two lists originate from the 1952 Immigration and Naturalization Act, but are updated at the State Department's discretion. She said she "didn't know why" South Africa — a democracy and an allegedly close ally of the United States — is on the list.

But Lerch claims that the implementation of these rules has changed. "There has never been a case quite like this," he says about Hu's referral. The State Department, he says, "can turn these things on and off as they choose. It is very clear that there are a lot of people at the State Department who are

Pay awards split Indian research world

[NEW DELHI] The awarding of higher salaries and financial perks to government scientists in India's departments of defence, atomic energy and space — but not other agencies or universities — has divided the scientific community.

The latest controversy has been triggered by an order issued last month sanctioning special monthly pay of Rs2,000 (US\$47) and an annual professional 'update' allowance of Rs5,000 for all scientists in these three strategic departments. The benefits will be backdated to January 1996.

Many scientists were already upset by the government's decision last November to deny them the fast-track promotional ladder available to defence, atomic and space scientists (see *Nature* 396, 299; 1998).

Critics say the initial move, introduced after India's nuclear tests last May, sent the wrong signals to the scientific community, as it appeared to suggest that good work means developing bombs, missiles and satellites rather than producing a vaccine or crop variety.

"The government has created a new caste of super-scientists," says S. R. Valluri, former director at the Council of Scientific and Industrial Research.

The initial order said the incentives were given in view of "the role played by them in the development of high technology and systems for strategic applications... and in order to attract, retain, inspire and motivate scientists to give their best contributions".

But Valangiman Ramamurthi, secretary of the Department of Science and Technology, admits that the move has instead "caused widespread resentment". He adds: "It is unfortunate that the scientific community is now fragmented. We [the secretaries of affected departments] have represented to the prime minister seeking a uniform package for all scientific departments and autonomous institutes."

Critics say the Bharatiya Janata Party government is equating scientific progress with bombs and missiles by declaring 11 May — the anniversary of the nuclear test — as Technology Day. **K. S. Jayaraman**

suspicious of all of the physical sciences.”

“We’re faced with a kind of hysteria,” he adds, contrasting the problems at this year’s physicists’ meeting in Atlanta with the last such meeting in the United States in 1987, when Ronald Reagan was president, and scientists from all countries were welcomed.

Scientists from countries labelled as ‘hostile’ who are already working in the United States have also felt the fallout from the Los Alamos case. However, they stress that it is the public mood, rather than the attitude of their scientific colleagues or managers, that worries them most.

“We would like to express our deep concern regarding the deterioration of the working environment of Chinese-American scientists and the scientific exchanges between the United States and China,” says a letter from Cheuk-Yin Wong, chairman of the 450-strong Overseas Chinese Physics Association, to APS president Jerome Friedman. The letter followed a discussion of 150 association members at the APS meeting in Atlanta and asks Friedman for a public statement of support for Chinese-American scientists.

Wong, a nuclear theorist at the Oak Ridge National Laboratory in Tennessee, is particularly concerned by media coverage of the Los Alamos incident. He cites an article in the 22 March issue of *Newsweek*, which described what it termed China’s “vacuum cleaner strategy” for using Chinese scientists in the United States for espionage. “There are statements in that article that will lead the general public to question the loyalty of Chinese-American scientists,” Wong says.

Some press coverage has also suggested that many Chinese scientists are in the United States because the Chinese communist government wants them to take secrets back home. In fact, China has become the biggest single source of scientific immigration to the United States largely because the US Congress passed legislation in 1992 giving Chinese students special rights to stay after the crackdown against student demonstrations in Tiananmen Square.

Wong says that for Chinese-American scientists at the nuclear weapons laboratories “the environment is very nervous”. But at other government laboratories, and at universities where most Chinese-American scientists work, this recent spate of China-bashing may have little effect.

“I have plenty of good things to say about the system here,” says a successful Chinese-born chemist at one civilian Department of Energy laboratory. He notes that he “can’t imagine the Chinese government doing the same” and allowing foreign scientists into its laboratories and universities. “It is true that people of my background have to work harder [than US-born colleagues], but I don’t think that is necessarily unfair.”

Colin Macilwain

Japanese researcher faces US charges over data

[TOKYO] The difference between Japan and the United States in their attitude towards intellectual property rights was highlighted last week when a Japanese researcher was accused of removing research data from a laboratory without proper authorization.

Yoichi Ito, a physician who was completing a research fellowship at the Mayo Clinic in Rochester, Minnesota, was charged with stealing research material three days before he was due to return to Japan.

He is accused of removing his research notes and materials from the clinic, as well as downloading research data from the clinic’s computer going back to 1990.

Ito, who had been working on gene sequencing in the clinic’s cartilage and connective-tissue laboratory, told the US magistrate in St Paul, Minnesota, that he “did not understand what was happening”, according to a report by the Associated Press.

But the Mayo clinic says he was warned on his arrival that the clinic held the rights to his research work, and that he would be required to return all materials produced during his term when he left.

Jesse Bradley, a spokesman for the clinic, says that all the regulations had been specified in Ito’s contract. “We are just trying our

best to get our research data back,” he says.

Although US institutions can impose stringent rules on the protection of such data, Japan has no regulations covering the rights to a researcher’s work outside the framework of standard patent and copyright laws.

“There are no legal requirements for researchers to return their research documents when they leave an institution,” says Yoshio Namba, of the intellectual property division of the Japan Science and Technology Corporation, a semi-governmental organization that runs a fellowship programme for overseas researchers to work in Japanese laboratories. “Such requirements are not included in the contract for overseas researchers either.”

“Research institutions do not have legal control over the researchers’ documents unless they are particular types of computer programs, with strict regulations on copyrights,” says Hiroko Saito, who is responsible for intellectual property rights issues at the Institute of Physical and Chemical Research.

Some scientists say that the different legal structure and general ‘culture’ of Japanese labs compared with those elsewhere could pose serious threats to collaborative research and exchange programmes. **Asako Saegusa**

Ethical failures block Los Angeles research

[SAN DIEGO] An interim research management team was sent last week from Washington to direct future research efforts at the troubled veterans’ hospital system in Los Angeles, where all research has been suspended by federal officials who are concerned about failures to correct long-running deficiencies in compliance with federal ethics rules.

The identified deficiencies were restricted to the sprawling West Los Angeles Veterans’ Affairs (VA) Healthcare Center. But they caused federal officials to shut down human and animal research at all VA facilities throughout the Los Angeles region.

Up to 1,200 research projects could be affected, according to VA officials. No patients can be enrolled in a

research project and no new protocol can start until scientists and administrators can document that all federal requirements will be met. Only studies in which the lives of patients or animals are at risk can continue, said officials.

Troubling questions about scientific practices at the West Los Angeles VA Healthcare Center — the largest of the VA hospitals — have been raised by the breadth of problems with the studies, the apparent lack of knowledge of senior federal officials about some unethical practices, and inadequate responses from management to years of administrative deficiencies.

Kenneth W. Kizer, the VA under-secretary for health in Washington DC, suspended all VA research in Los Angeles from 26 March, four

days after the National Institutes of Health’s Office for Protection from Research Risks suspended the assurance agreement under which VA scientists in Los Angeles conducted federally funded human studies.

In his letter suspending human and animal studies, Kizer wrote: “The lack of adherence to research policy and operational requirements is a very grave matter. Regrettably, facility management’s unresponsiveness now adversely affects individual investigators.”

VA officials in Los Angeles responded by replacing the acting director of research, appointing new chairmen to the human institutional review boards and animal review committees, and reassessing all committee membership. **Rex Dalton**

Primate study may yield new CJD clues ...

[PARIS] A study published last week that appears to show that primates are easily infected orally with bovine spongiform encephalopathy (BSE) may, if confirmed, have implications for the understanding of the new variant of Creutzfeldt-Jakob disease (vCJD) in humans.

The study could also provide a better and faster animal research model than those currently available. But researchers are angry that more extensive primate experiments, recommended to the European Commission in 1996 by one of its expert committees, have not been supported (see below). This reluctance, argue some scientists, stemmed from opposition in a number of member states to research using primates.

Two young *Microcebus murinus* lemurs, fed 0.5 g of cattle brain infected with the agent that causes BSE and autopsied five months later, were found to have prion infections, despite showing no outward clinical signs of the disease. Three control animals showed no signs of infection.

The group also autopsied two symptomatic and 18 apparently healthy large monkeys and lemurs from French zoos that had eaten animal feed produced in the United Kingdom. All were found to be infected.

The work was published in the 30 March issue of the *Proceedings of the National Academy of Sciences (PNAS)* by a Franco-American team including Noëlle Bons of the University II of Montpellier, Paul Brown of the US National Institute of Neurological Disor-



Model subject? Lemurs may be good animals for studying spongiform diseases.

ders and Stroke in Bethesda, Maryland, and D. Carleton Gajdusek, the 1976 Nobel prize winner for his work on prions, now at the Institut Alfred Fessard near Paris.

Charles Weissman, from the University of Zürich, in Switzerland, points out that a major caveat in the study is that infection was assessed by immunostaining of proteinase-resistant prion protein, rather than by Western blotting of abnormal prion protein. Fixed brain samples which cannot be tested using a blot were taken at the time, explains Bons, adding that ongoing experiments will use frozen samples and Western blotting.

At the same time, Weissmann — who chaired the committee that advocated more

primate studies in 1996 — says that he hopes the new findings may encourage more interest in these. The findings, if confirmed, could have “very worrisome implications” for humans, he adds, as they suggest that primates closely related to us may be highly susceptible to oral transmission of BSE.

Dominique Dormont, whose group demonstrated the first transmission of BSE to a macaque (see *Nature* 381, 743; 1996), points out that *Microcebus murinus* may be a valuable new animal model. This tiny species weighs just 100 grammes and has a short lifespan of eight to ten years, making it much more suitable for research than macaques and other larger, longer-lived primates.

According to Bons, another lemur from another experiment has fallen ill with symptoms of spongiform encephalopathy one year after being fed contaminated cattle brain. The short time to incubation and illness suggest the species would be ideal for research purposes, she says, warning at the same time against the dangers of extrapolating results directly to humans.

In the experimental *PNAS* study, infection was found throughout the tonsils and peripheral tissues, and confirms an oral route of transmission of BSE, says Dormont. The agent appears to pass through the digestive tract, across the spleen, and up the spinal cord into the brain, says Brown.

Bons argues that the results also suggest that nervous degeneration and wide peripheral infection occurs early in the incubation phase, raising the prospect that in humans asymptomatic individuals could be carrying high levels of infections — which might have implications for secondary transmission. “We know nothing of the kinetics of vCJD in humans [where living individuals cannot be autopsied],” says Brown, arguing that primate studies could therefore yield new information on this.

The dose of brain fed to the lemurs is equivalent to a 70 kg man eating a dose of 500 g. The larger experiments needed to establish the minimum dose needed for infection could not be carried out, however. This is “unfortunate,” says Weissmann, pointing out that his committee called in 1996 for precisely such experiments, as well as ones on maternal transmission.

Bons is also studying two baby lemurs born from infected parents. She says she is collecting samples to establish whether and how maternal transmission occurs.

The *PNAS* paper contains another potentially controversial element. According to Bons, the only meat in the zoo primates’ food was British ‘cretons de bœuf’, a meat residue approved for human consumption. A fuller inquiry into the source of infection is needed, she says.

Declan Butler

... as researchers fume over blocked research

[PARIS] Dominique Dormont, a prion expert who is head of the French government’s spongiform encephalopathy advisory committee, is — like many BSE/CJD researchers — dismayed at what he claims are the years lost because of an apparent lack of political support for work using primates.

Dormont says that a proposal for experiments on macaques, submitted in 1996, was not approved because some states — including the United Kingdom — opposed it on animal-welfare grounds.

Large-scale primate experiments can only be carried out at a continental level, says Dormont, because of the high costs and

logistics. He says that some countries felt such experiments would be a waste of money, as, by the time their results came through, the results of human exposure to BSE would already be beginning to appear.

Paul Brown, of the US National Institute of Neurological Disorders and Stroke, one of the authors of the *PNAS* paper (see above), adds that he advocated similar experiments in the United States at roughly the same time. But he claims that government safety restrictions on BSE and vCJD materials have all but halted research.

“I can understand that they don’t want every Tom, Dick and Harry working on

these agents,” says Brown. “But the regulations are blocking all research in the handful of labs who have forty years experience handling these agents.”

Noëlle Bons, from the University II of Montpellier, another author of the paper, wrote to Edith Cresson, the then European commissioner for research, in 1998, presenting her with the preliminary results of the *PNAS* paper. She also complained that she had heard nothing regarding a research proposal on BSE she had submitted the previous year. Bons claims that, despite meeting a member of Cresson’s staff, she remains in the dark as to support for her project.

D.B.

Anger at NZ plan to target social goals in research

[SYDNEY] Scientists in New Zealand are facing another major restructuring of government research funding, following extensive reforms eight years ago.

New measures announced last month from the country's Foresight programme aim to redirect half of the NZ\$600 million (US\$320 million) science budget to supporting research relevant to achieving social in addition to economic goals.

The reforms, which will affect New Zealand's NZ\$300 million Public Good Science Fund, have angered many in the academic research community, not just because of the speed and scale of the changes, but also owing to a decision to reduce the use of academic peer review to allocate funding.

Groups made up of industrialists, scientists and community organizations will be invited to submit ideas for 'portfolios' of medium-term projects lasting five to six years. Final decisions on funding will be made by the government following advice from the Foundation for Research, Science and Technology (FRST), which administers the so-called Public Good Fund. Previous allocations from this fund were typically for single projects worth around NZ\$500,000 over three years.

The priorities of the current Foresight programme have been replaced by 17 'outcomes' for research that are expressed as generalized, social goals. These include:

- A culture of innovation, anticipation and creation of new markets;
- Empowered individuals and communities;
- High health status;
- Healthy, diverse, resilient ecosystems;
- Maori development, distinctive and positive cultural identity; and
- Self-determination and ethical principles.

The government says the reforms are not intended to relax efforts to forge partnerships between universities and industry. Prime minister Jenny Shipley has directed Max Bradford, the new minister for tertiary education, to collaborate with the minister for research, science and technology, Maurice Williamson, and the government's Crown Research Institutes to direct universities to align themselves closer to industry.

But biomedical researchers are particularly concerned about the reforms. They are lobbying the Health Research Council not to allow its budget to be influenced by the new Foresight priorities or the new method of allocating funds.

Janet Grieve, president of the New Zealand Association of Scientists and a senior government researcher, last week

wrote to the prime minister and the cabinet complaining that the "unspecified methodology [for funding allocation] is causing great disquiet and anxiety among scientists".

Michael Berridge, the association's secretary, is another prominent critic. He argues that the reforms are "based on pure faith". And he adds that more weight should be given to scientific merit than relevance to society in allocating research funds.

Paul Callaghan, a leading physicist at Massey University and president-elect of the academy council of the Royal Society of New Zealand, is also concerned. Callaghan believes that core disciplines such as physics will suffer if they can only "piggy back on to an applied portfolio". Criticism has also come from Michael Irving, vice-chancellor of Victoria University of Wellington.

But the reforms have been welcomed by the leaders of the government-owned Crown Research Institutes, which derive most of their income from the Public Good Science Fund. Support has also come from the FRST.

Stephen Thompson, the foundation's chief executive, claims that the new priorities will "encourage creativity" and are well suited to science in a small country. But he acknowledges that the new model has no parallels in other countries.

The reforms carry the enthusiastic support of Prime Minister Shipley, who told parliament that they are aimed at making science more relevant to the wider community. The government's longer-term aim is to use the reforms to illustrate to politicians the need to increase overall levels of research funding, say proponents of the measures.

But Shipley, who leads the conservative National Party, is in a politically precarious



Bradford: work with industry.

position. She heads a minority administration, and in February survived by just two votes a no-confidence motion in parliament.

Meanwhile, Jane Kelsey, president of the Association of University Staff, which is campaigning against parallel proposals to restructure universities, believes that Bradford may be cooler in his support for that plan

than the author of the proposals, his predecessor, Wyatt Creech (see *Nature* 396, 502; 1998). Bradford has delayed legislation for the time being. Creech was recently appointed deputy prime minister in a ministerial reshuffle.

Peter Pockley

Brazilian scientists team up for cancer genome project

[SÃO PAULO] Brazilian researchers have entered the competitive field of human genome sequencing with the signing of an agreement between the state funding agency of São Paulo (FAPESP) and the US-based Ludwig Institute for Cancer Research.

Each will contribute US\$5 million to a two-year Human Cancer Genome Project. According to FAPESP, the programme is "aimed at providing sequences from genes expressed in tumours that are important within the context of public health in the state of São Paulo".

The project will sequence and analyse short DNA fragments created from the central coding portions of human genes. Although a US patent is being sought for the technique used to generate these expressed sequence tags (ESTs), the sequences will be freely available on the Internet. "No sequences will be patented. All the data will be promptly published," says Ed McDermott Jr, president of the Ludwig Institute, who visited Brazil to sign the agreement.

The programme follows on from the Organization for Nucleotide Sequencing and Analysis (ONSA), a network of 30 laboratories in the state of São Paulo now in the final steps of sequencing the complete genome of the plant pathogen *Xylella fastidiosa*. The groups will build upon their experience with this pathogen, which causes many economically important plant diseases, notably citrus variegated chlorosis, which poses a major threat to São Paulo's orange farming (see *Nature* 389, 654; 1997).

ONSA is a 'virtual' institute that links the sequencing laboratories, keeping down costs and red tape. The acronym, which sounds like the word *onça* (jaguar) in Portuguese, mimics the Institute for Genomic Research (TIGR), according to José Fernando Perez, FAPESP's scientific director.

Five centres will carry out the sequencing, each helped by four other labs. The centres will be at the chemistry institute, the faculty of medicine at São Paulo, and the faculty of medicine at Ribeirão Preto, all from the University of São Paulo; at the Paulista School of Medicine, São Paulo; and at the Hemocentro of the University of Campinas. The programme aims to generate between 500,000 and 750,000 EST sequences, and about 200 million bases of human genome sequence.

The project will be monitored by a four-member steering committee, composed of Marcelo Bento Soares of the University of Iowa, John Sgouros of the Imperial Cancer Research Fund in London, and Webster Cavenee and Richard Kolodner of the Ludwig Institute in San Diego. Ricardo Bonalume Neto

Biometrics group counters privacy fears

[WASHINGTON] A newly formed US industry group has announced a set of principles that, it says, should protect users of biometrics against privacy abuses. The move is a bid to pre-empt attacks from civil libertarians and politicians anxious to capitalize on public anxiety about data privacy.

The International Biometric Industry Association (IBIA) says its code calls for legislation to control government use of biometrics. But it would leave private industry unregulated except for voluntary adherence to the principles. "If the end-users and customers of the technology are cognizant of the [privacy] issues, this is clearly an area that can be self-regulated effectively," says Richard Norton, executive director of IBIA.

A biometric is an electronic code representing some unique physical feature — commonly a person's hand geometry, iris pattern, fingerprint or face — that is used to verify that person's identity against a reference code provided by the person and stored digitally.

Norton says the group was motivated by about 150 privacy bills introduced in the last Congress — and 38 so far in this one — that would affect biometric technology makers and marketers, often adversely. Rather than face the consequences of such bills becoming law, or of having to deal with a patchwork of state laws in the absence of federal action, IBIA is seeking pre-emptive federal legislation limiting government use of biometrics.

Privacy advocates say that digitized identification systems are easily abused by government and industry. Computerized biometric information can be manipulated and transferred swiftly and easily. A biometric is unique and cannot be forged like a signature, stolen like a password or lost like a card. Because of its near-infallibility, they argue, industry or a 'Big Brother' state could use an individual's biometric as an all-purpose identifier that could be used to deny benefits, restrict travel, or even obliterate the individual as a state-recognized entity.

"A majority of citizens have something to fear from biometrics because information that identifies you intimately in one area of your life can easily be used to adversely score you in another," says Simon Davies, director of Privacy International, a London-based group that monitors surveillance by governments and companies.

Davies says the potential for abuse is particularly dangerous in countries with authoritarian regimes. He complains, for instance, about IBM's recent work with the Peruvian government to digitize fingerprints to prevent fraud in applications for national identity cards.

"What lies around the corner is the same biometric being used for social security,



Private eye, public information: security systems such as those that scan irises could be abused.

police purposes... a whole range of other purposes."

Barry Steinhardt, associate director of the American Civil Liberties Union, says biometrics present a real danger of the development of a 'surveillance state'. He says: "We have grave concerns about how [this technology] is going to be used. [Will it] make it impossible for people to go about routine business in an anonymous way without being subject to surveillance or having more and more data collected about them, either by government or by private industry?"

But the IBIA, which represents 15 companies with revenues of \$35 million, says its members are being targeted by a misinformation campaign. It argues that the technology is safe, user friendly and a near-perfect defence against identity theft, cheque fraud and other privacy abuses.

If the critics understood what biometrics do, they would "see this as a tool for protecting privacy rather than running around saying the sky is falling," says John Siedlarz, IBIA vice-chairman, who is president and chief executive of IriScan, a company based in Marlton, New Jersey.

The industry says that biometrics enhance rather than undermine personal data security, providing a system that is much less vulnerable to fraud and abuse than, say, the use of PIN numbers at banks or passwords to access computer networks.

"You can't take the code that's been generated by the minutiae of a fingerprint or hand geometry and reverse-engineer that to show who I am. So it is a very secure way of locking up information," says Norton.

The IBIA principles say that biometric data must not be "released without personal consent or the authority of law". They call for industry to develop "policies that clearly set forth how biometric data will be collected, stored, accessed and used", and that preserve individuals' rights to limit the distribution of their data beyond the original purpose.

The principles say that "clear legal standards" should be developed to define and limit the conditions under which government agencies can acquire and use biometric data. And they call for public and private sectors to adopt "appropriate managerial and technical controls" to protect databases containing biometrics.

But Davies calls the principles "a Sesame Street privacy code" which, like other voluntary industry codes, is "unenforceable, illusory, counterproductive" and likely to be ignored by governments.

Similarly Robert Gellman, an independent privacy consultant in Washington, says the code is "a very casually written document without a clear understanding of privacy principles or fair information practices".

"The principles could be a lot clearer and stronger, and they're not. This is the usual American approach of saying you're going to do something about privacy, only not in a way that has any teeth." **Meredith Wadman**

Hand and eye security systems in growing use

[WASHINGTON] Biometrics have been widespread for some time in sectors such as law enforcement and the nuclear power industry, where hand geometry is used to control access to secure facilities. But they have begun in the last five years to be used in ways far more likely to be encountered by ordinary people.

Since 1995, for example, the US Immigration and Naturalization Service has enrolled more than 80,000 travellers in a system that allows them to jump lengthy

queues at airports by scanning their hand geometry.

Companies are using fingerprint imaging devices to serve as passwords for computer networks. And welfare programmes from Connecticut to Los Angeles County are using biometrics to deter welfare fraud.

If privacy advocates are anxious about the implications of biometrics, some members of the public may be less concerned. In the United Kingdom, the Nationwide Building Society

and NCR Corporation last year tried out an iris scanning system with 1,000 customers at Nationwide's Swindon branch.

Instead of using PIN numbers or signatures at the cash machine or bank counter, customers' irises were recognized by cameras which gave them access to their accounts. Some 91 per cent of participants favoured the system over the use of PIN numbers or signatures, according to the Pegram Walters Group, which carried out the research. **M.W.**

Russia to focus science on high-tech goals

[MOSCOW] Russia's minister of science and technology, Mikhail Kirpichnikov, has put forward a package of measures which, he argues, need to be introduced if Russia is to use its scientific achievements to boost its capacity for technological innovation.

The package includes economic and legislative means for stimulating investment in science-based production; insurance cover for such investment; and financial support for enterprises that engage in such production. Resources would be directed towards projects that use high technology to achieve commercial goals, as well as contributing to social priorities and national security, and helping to reduce imports.

The new measures' goal was endorsed in President Boris Yeltsin's annual message to the Federal Council (the Russian parliament) last week. Yeltsin described science and technology as "strategic prerequisites for the growth of the national economy—and hence the improvement of the quality of life".

Kirpichnikov's ideas have already been endorsed by the cabinet, but his strategy differs significantly from previous approaches to the administration of Russian science.

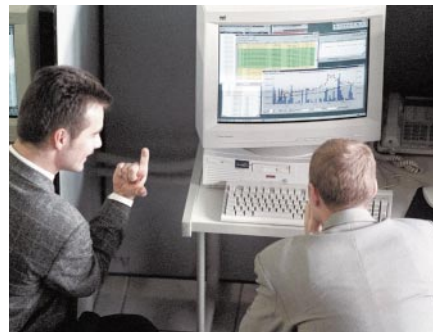
First, it deals only with a limited period of less than one year. "This is because it is difficult nowadays to make plans for a longer perspective," explains Kirpichnikov, who is a full

member of the Russian Academy of Sciences. Second, his ministry is, for the first time, basing its approach not on pointing out the extra money science needs — a strategy that has met little success in the past — but on asking what science can contribute to the economy.

"To achieve the standards of life typical for developed countries we need to increase our gross national product," says Kirpichnikov. "There are three ways of doing this. Russia is too big to rely totally on selling its raw materials — it takes a few thousand kilometres to transport oil or gas to the Russian frontiers — and we cannot afford new technologies. The only option left is through innovation, based on the fact that Russia still has powerful fundamental science and has managed to keep its intellectual potential in spite of the fact that in the last 10 years it lost half of its scientists."

Kirpichnikov argues that the way out of the economic crisis is by using Russia's scientific and intellectual potential to create high-tech production, selling patents and know-how. But he points out that two obstacles stand in the way: the lack of strong demand from industry and agriculture, caused by the difficult economic situation, and an underdeveloped infrastructure for innovation.

It is to overcome the first barrier that Kirpichnikov has suggested the measures to be taken at government level. Another sugges-



Technology's our one hope: traders track the economy at the Moscow stock exchange.

tion is that the stable functioning of scientific organizations could be ensured by their winning orders from the state for high-technology products through competitive bidding.

Kirpichnikov adds that the science ministry is seeking to overcome the second obstacle by setting up a series of Innovation-Technological Centres (ITCs). These bring together small companies able to transform scientific results into competitive industrial production. They are situated in half-empty or incomplete buildings of factories or scientific institutions, and modestly supported by local and federal budgets.

There are now 18 ITCs, with more than 250 small companies and 7,000 scientists, and 17 more centres are to be set up. ITCs have shown they can speed the innovation process by up to three times while halving costs. They return the initial budget funding within one or two years by paying taxes.

The annual cost of products sold per scientist linked with an ITC equals US\$15,000–25,000 — higher than average for Russia. But Kirpichnikov points out that each rouble invested in ITC activities can bring in five to 12 roubles a year.

Kirpichnikov considers the ITCs to be only the first link in the innovation chain. This year, the science ministry will also create two or three Federal Centres for Science and High Technologies, which will bring together around a leading scientific organization universities, design offices, standardization and certification centres, as well as factories.

Such centres will concentrate their efforts in fields in which small companies are unable to solve the problems, such as aircraft production, shipbuilding and energy production. Federal centres based on the country's biggest enterprises are expected to surpass the production of the ITCs by 10–100 times.

"All these will allow us as soon as the end of this year to make a real move towards the high-technology economy," says Kirpichnikov. He promises that in 2000 the government will increase science funding to no less than 4 per cent of all state spending, as promised by Yeltsin in 1996.

Carl Levitin

US spallation project 'running into trouble'

[WASHINGTON] A prominent Republican Congressman says that management of the US Spallation Neutron Source (SNS) at the Oak Ridge National Laboratory in Tennessee is "in turmoil". He wants construction of the \$1.4 billion facility to be halted until the management problems are fixed.

James Sensenbrenner (Republican, Wisconsin), chairman of the Science Committee in the House of Representatives, visited the project last month before publishing a blistering critique of the Department of Energy's management of it. The SNS is the largest scientific user facility that the department has attempted to build since the Superconducting Super Collider project, abandoned in Texas in 1993.

"SNS project management is in turmoil, spending is lagging, and project cost and schedule estimates have not been fully developed," says Sensenbrenner. He adds that the energy department's effort to share responsibility for the project among five laboratories requires simplification.

Sensenbrenner's attack on the project — which he says he still supports — follows a difficult winter for the spallation source. Events culminated in the replacement in February of the project director, Bill Appleton of the Oak Ridge laboratory, by

David Moncton of the Argonne National Laboratory in Illinois.

It was not immediately clear if Sensenbrenner means to try to cut off the \$214 million that the energy department has requested for SNS construction in the 2000 financial year, which starts in October, or if he just wants to ensure that project management is quickly improved.

"When the chairman of a congressional committee makes a statement like that, you have to take it seriously," says Martha Krebs, head of the department's science office. "It could mean that there is a real problem [with funding], or it could mean that there is room for discussion."

Krebs says that Moncton will present a full technical review of the project to senior department officials this week. She warns that it could be "almost impossible" to continue if construction funds were halted and money only appropriated for research next year, as Sensenbrenner suggests.

Tennessee is the home state of Vice-President Al Gore, and Sensenbrenner's critics believe he wants to make trouble as Gore prepares to run for the presidency in 2000. But the project also has powerful Republican supporters, including Senator Bill Frist of Tennessee.

Colin Macilwain

'FLAT Earthers' in battle with creationism

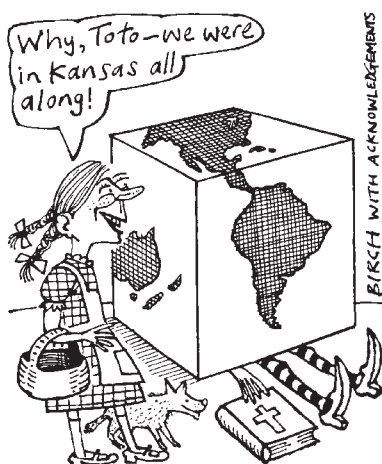
[SAN DIEGO] A group of US scientists and educators is using parody to fight creationism in a school board election in Kansas. The group argues, for example, that the teaching of 'round Earth theory' in schools should be questioned, as the Bible makes reference to the world having four corners (Revelations 7:1).

When creationists started an organization to diminish the teaching of evolution in schools in Lawrence, Kansas, the scientists formed FLAT — Families for Learning Accurate Theories. FLAT has posted its 'Biblical' views on the Internet.

"We don't necessarily all believe the world is flat," says Philip G. Kimball, FLAT's treasurer, an author with a masters degree in education from Stanford University. "Another possibility is a tetrahedron. The group isn't settled on the Earth's final form."

With tongues firmly planted in cheeks, FLAT members comment that the Bible says that π is 3, not 3.14 (1 Kings 7:23), and that studying foreign languages is 'unbiblical', as God created many languages to prevent people from recreating the Tower of Babel.

In an interview with *Nature*, Kimball insisted on adhering to the satirical line. In contrast, Adrian L. Melott, a cosmologist at



the University of Kansas in Lawrence and another founder of FLAT, acknowledged that parody was being used because "reasoned argument with creationists has failed".

"The dialogue has given the public the idea that there are two sides to the story of equal weight," said Melott. "Scientifically, that isn't the case."

FLAT was formed to counter a group called Parents for Objective Science and History (POSH), formed by Ellen L. and Joel M.

Barber, who objected to their five-year-old son being taught in kindergarten that dinosaurs lived millions of years ago. As creationists, the Barbers say they believe the Earth was formed 10,000 to 60,000 years ago.

With a campaign under way for last Tuesday's (6 April) election for the board of the Lawrence Public Schools, the Barbers and POSH sought to persuade candidates to advocate teaching evolution as theory.

When FLAT parodied their efforts, Ellen Barber said she felt "ridiculed", adding: "They are trying to discredit our views. If they discredit God or the Bible, we are discredited." Melott denies this, saying that religion has its appropriate place in schools — but not as a so-called science.

Ironically, say Melott and Kimball, their FLAT campaign has brought them ridicule as well, with hate mail posted on the Internet and derision at home, because many people seem to have missed the joke. "My wife wants to use her maiden name for a couple of weeks," said Melott.

Humour aside, the issue of teaching creationism in schools is a serious business in Kansas. Creationists are lobbying the state board of education to include their view in statewide scientific standards. **Rex Dalton**

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They should include a CV, a discussion of relevant experience, and an indication of their specific ambitions for *Nature*. Applications should be sent as soon as possible and no later than 16 April.

France's biomedical agency launches plan to combat misconduct

[PARIS] France's national biomedical research agency, Inserm, has set up a Delegation of Scientific Integrity charged with "evaluating the reality of allegations of bad scientific conduct" and making recommendations for dealing with the problem. The new body will consist of a national scientific committee and local 'mediators'.

The delegation will be headed by Martine Bungener, head of the agency's Laboratory of Medicine, Science, Health and Society, and will be able to call in international experts if needed. The delegation has also been charged with liaising with similar European bodies to come up with recommendations to promote "professional ethics of scientific publication". Inserm has also launched a scheme to encourage 'good practice guidelines' in its laboratories, to "assure the quality, traceability and integrity of data".

The move follows widespread criticism of the agency's handling of allegations of misconduct at one of its laboratories. It failed to carry out its own investigation of the affair, while the science ministry — apparently unenthusiastic from the outset — quietly dropped the issue (see *Nature* 391, 519–520; 1998). The French public prosecutor subsequently opened a formal judicial inquiry into the allegations.

Senators attack budget cut for military research

[WASHINGTON] Nine US senators have written to William Cohen, the defence secretary, objecting to administration plans to cut funding for military research and development by six per cent next year. The senators, who include prominent figures from both parties, complain that military research spending has already fallen by 30 per cent in the past six years.

The senators say it is short-sighted of the administration to boost general military spending next year while cutting research and development still further. They point out that half of the US Nobel prizewinners in physics or chemistry since 1950 have been funded by the Department of Defense.

Russia begins tests on 'globe' tokamak

[ST PETERSBURG] Russian scientists have embarked on experiments to generate and maintain a high-temperature plasma in the small spherical 'Globus M' tokamak reactor in St Petersburg. The experiments have been paid for by Japan and the European Union.

The reactor, which resembles a giant globe and weighs five tonnes, was built at a St

Petersburg aircraft works as part of a programme to convert military facilities to civilian use. An additional US\$150,000 has been allocated to continue the project beyond the initial ignition stage.

Japan gets set to build its own spy satellites

[TOKYO] Japan will be able to rely entirely on domestic technology to develop reconnaissance satellites, chief cabinet secretary Hiromu Nonaka announced last week. The satellites, scheduled to be launched by 2002, are intended to improve the nation's ability to gather security information following last year's firing of a North Korean missile over Japanese territory.

Because of Japan's lack of experience in developing such satellites, the ruling Liberal Democratic Party had considered seeking technological help from the United States (see *Nature* 396, 401; 1998). But the government now says the National Space Development Agency will be able to provide the required technology.

US grants for work on Large Hadron Collider

[WASHINGTON] Northeastern University in Boston, Massachusetts, has received a \$20 million grant to lead US university participation in the design and construction of the Compact Muon Spectrometer, one of the two particle detectors at Europe's Large Hadron Collider (LHC).

The five-year grant from the National Science Foundation is the largest element of the \$80 million that the foundation will spend to support university scientists involved in LHC construction. It goes to Stephen Reucroft of Northeastern's physics department, who will collaborate with groups at eight other physics departments. The US Department of Energy is already supporting scientists at its laboratories who are participating in the construction of the muon spectrometer and of ATLAS, the other LHC detector.

China receives funds to phase out use of CFCs

[LONDON] China, the world's largest producer of chlorofluorocarbons (CFCs), is to be given US\$150 million to close down its CFC-producing facilities over the next 10 years. The amount was approved at the end of last month by the committee that administers the Multilateral Fund of the Montreal Protocol on substances that deplete the ozone layer.

The committee also agreed to give an additional \$30 million for projects that phase out CFC-production in other developing countries. It also approved a \$7 million contribution to that end from the United

Nations Environment Programme. More than \$900 million have now been spent on such projects.

Research reactor gets go-ahead in Australia

[SYDNEY] Australia's environment minister, Robert Hill, gave his approval last week to the construction of a research reactor to replace the HIFAR model operated for 40 years by the Australian Nuclear Science and Technology Organization (ANSTO) at Lucas Heights, an outer suburb of Sydney. The decision followed satisfactory assessments of the project's likely environmental impact by three external bodies and Hill's department after the project was approved in principle by the cabinet 19 months ago, subject to a satisfactory outcome (see *Nature* 389, 109; 1997).

A final go-ahead from science minister Nick Minchin to call contracts for the pool-type reactor is expected shortly. At a total cost of A\$300 million the reactor will be by far the largest public investment in a science and technology facility in Australia. Construction is due start in 2002, with operational replacement and decommissioning of the old reactor in 2005.

DNA chips 'bring risk of genetic discrimination'

[LONDON] DNA chip technology could lead to genetic discrimination, even eugenics, unless appropriate regulations are put in place, according to a paper in the *Journal of Medical Ethics* (25, 200–203; 1999). According to Wolfram Henn of the Institute of Human Genetics in Homburg-Saar, Germany, DNA chip technology will be capable of analysing an individual's genetic make-up within a few years.

"It is a blessing for clinical medicine," Henn writes. But it also "provides employers and insurers with ethically questionable parameters for aptitude tests".

US bill offers tax breaks for developing vaccines

[WASHINGTON] US politicians are seeking a tax break for companies working on vaccines against tuberculosis, malaria and HIV. A new bill would provide a 30 per cent research and development tax credit for research into vaccines against any disease that kills more than one million people a year, and for work on developing microbicides.

The bill was introduced in the House of Representatives by Congresswoman Nancy Pelosi (Democrat, San Francisco) and Congressman Charles Rangel (Democrat, New York). A Senate companion bill is expected to be introduced by John Kerry (Democrat, Massachusetts).

Search for a quick getaway from Mars

Sir — I was heartened to see your article on Carlo Rubbia's proposed propulsion system to send humans to Mars (*Nature* 397, 374; 1999). I emphatically agree with Rubbia that fast missions will be required to reduce to manageable levels the dose of galactic cosmic rays to which the crew would be exposed. Calculations at the Los Alamos National Laboratory in New Mexico indicate that the crew could receive their entire allowable lifetime dose of 200 cSv during a three-year mission. More than half of this dose would come from radiation on the surface of the planet.

Rubbia is also correct in stating that a new, advanced, high-performance propulsion system would allow fast missions with return trips of about one year. Such a mission is well within human experience — 500 days trapped in a pup tent

on the surface of an unknown world is not.

I must disagree with the article and Rubbia with respect to the stated performance of the NERVA nuclear rocket. The US space agency NASA is currently trying to use chemical rockets, such as those on the Space Shuttle, in its mission studies, which would allow round-trip missions of three years. During the 1985 Manned Mars Mission Study by the Los Alamos laboratory and NASA, the impact of using a NERVA rocket was reinvestigated. Los Alamos also proposed redevelopment of the NERVA engine during the Space Exploration Initiative. NERVA was tested by Los Alamos in the 1960s, having a specific impulse of 850 seconds. Such an engine would allow a Mars mission of 434 days for the same mass in low Earth orbit that the chemical engines will require. This is proven technology.

Los Alamos has also been studying a concept, the gas-core nuclear rocket, that would have a specific impulse equal to the concept of Rubbia, 3,000 seconds. Such an engine would enable a nine-month mission to Mars. In addition, the performance of the rocket allows the spaceship to carry shielding against cosmic rays. Estimates show the dose to the crew would be reduced by more than a factor of ten.

Rubbia's concept is as viable as the gas-core rocket concept. To send humans to Mars will require something with the performance of these engines. I eagerly await the results of the design analysis of the Rubbia concept with regard to engine mass, radiator mass, power requirements and thermal loading.

Steven D. Howe

Los Alamos National Laboratory, New Mexico, USA

Sartorial simplicity is knot what it seems

Sir — As a former denizen of the British boarding-school system, I was delighted to find that simpler and mathematically rigorous methods exist for knotting a tie (*Nature* 398, 31–32; 1999).

I wondered, however, whether experimental testing might reveal unexpected flaws in the modelling process.

I began with the three-move reverse-start knot, which is one move shorter than the four-in-hand knot I learned as a schoolboy. All was well. Until... as I went to remove the experimental apparatus (my old-boy tie), I discovered to my horror that the 'simpler' knot left me with an overhand loop. The four-in-hand (and all knots with an even number of moves, including the Windsor and half-Windsor) leaves no such lingering trace requiring further engineering solutions.

I have reached three major conclusions from my experiment. (1) I shall generally stick with the method I learned in my Dickensian past, unless (2) I am late for an appointment requiring a tie, and the fraction of a second gained at the outset outweighs the delayed cost of undoing an overhand loop at the end of the appointment. And (3) a model is rarely complete until it has been field-tested in a variety of contexts.

Incidentally, many inhabitants in this part of North America have circumvented the entire suite of theoretical and applied

problems entailed in knot tying by adopting the bolo tie.

David B. McDonald

Department of Zoology and Physiology, University of Wyoming, Laramie, Wyoming 82071-3166, USA

Sir — I have spent some time in front of a mirror studying the conclusions of the Fink–Mao analysis of tie knots. As my profession of lexicography involves close attention to terminology, I was interested to note the authors' restraint in not proposing names for the unnamed knots.

The first knot in the table, {3, 1}, which is even simpler than the common 'four-in-hand' {4, 1}, is presumably a 'three-in-hand'. The 'inside-out' knot {7, 2}, after its introductory move, in fact consists of the same moves as the half-Windsor {6, 2}, but inverted left to right. It might be referred to as the 'inverse half-Windsor'. The more complex knot {9, 3} bears the same relationship to the Windsor, and so might be called the 'inverse Windsor'.

The knot {7, 3} concludes with the same winding sequence as the half-Windsor, but begins with an 'LC' move reminiscent of the full Windsor, and reproducing the beginning of the Pratt knot {5, 2}. I suggest it must be either a 'three-quarter-Windsor' or a 'Pratt-Windsor'.

That leaves the curious {8, 2}, a superficially elegant knot which (as an Oxford man) I suggest calling the 'Cambridge', and the monumental {9, 4}, for which the name 'Cavendish' presents itself, in honour of the originating institution.

Jeremy H. Marshall

54 North Hinksey Village, Oxford OX2 0NA, UK

Grant agencies must be seen to be fair

Sir — Each year the International Agency for Research on Cancer (IARC) in Lyons, France, announces a competition for training fellowships. As a rejected candidate in last year's round, I would like to draw attention to what I consider to be a non-transparent system of selection, which fails in its responsibility to give feedback to young scientists trying to establish a career.

I was given only seven days notice of an interview which was conducted by only one person. Yet, despite this short notice, I had to wait three months before being told — by fax — that my application was unsuccessful.

I immediately asked the IARC for the reason for my rejection. I was told that it was against agency policy to give such information. I then asked for more precise information about the list of applicants, the scoring each received and, again, the reason for my failure to be shortlisted. After some delay I was told that this information was confidential.

Obtaining a research fellowship is a very important step in the career of a young researcher. I consider it fair that unsuccessful applicants should be allowed to understand the reasons for their exclusion and the evaluation criteria used. And interviews should be conducted more openly. IARC, and all grant-giving agencies, should employ more transparent practices.

Stefano Parodi

Ligurian Cancer Registry, National Cancer Institute, Largo R. Benzi 10, 16132 Genova, Italy

Revealing the hidden costs of research

How should universities account for the money they receive from governments? The answer is not as simple as it may at first appear. There are valuable lessons that other countries can learn from the US experience.

Robert M. May and Stuart C. Sarson

How effectively are science budgets deployed? Exactly how are funds spent in the laboratory? The pressure for enhanced transparency of science spending is increasing. In the United States, for example, there has been a long-running and occasionally fractious debate about what is reasonable to count as the 'indirect costs' of research. And one of the conditions of extra money allocated by the UK government last year for science and engineering was that the research sector would improve the transparency of how the money is used. How should this be achieved?

A key factor in financial transparency is the accounting of indirect costs, a source of much debate and friction. In particular, attribution of indirect costs requires individual recipients of funding to make a notional apportionment of their time between research, teaching and administration — activities which, in a happily managed university, are seamlessly interwoven. How these issues are addressed in the United States is relevant elsewhere, because the US system for identifying the indirect costs of research within a large and diverse higher-education sector has evolved over the past half-century. We hope that what follows will be useful, even provocative, to US researchers as well as to those in other countries who are, or soon will be, wrestling with similar questions.

Indirect costs

The US system is based on the principle that the federal government reimburses universities the full costs associated with any research that is carried out for it. This system has its origins in the aftermath of the Second World War, when the principle on which defence contracts were based was transferred into civil research.

The costs of a research project are split into the direct costs — which can be directly attributable to that project — and the indirect costs, such as administration, building operation and maintenance, and library support, which are not easily attributable to individual projects. Clearly, for universities to operate sustainably they must receive payment for these indirect costs. Although easy to state in the abstract, this principle is not at all straightforward to put into practice.

The first issue is to identify the indirect costs. Despite specific guidelines, this requires negotiation, during which feelings can run high. Universities must each submit a proposal based on historical costs to one of

Table 1 **Negotiated indirect-cost rates for selected US universities**

Public		Private	
Institution	Rate	Institution	Rate
Univ. California, Berkeley	49.9	Brown Univ.	60
Univ. California, Los Angeles	49	CALTECH	57.45
Univ. Maryland	48	Univ. Chicago	53
Univ. Michigan	52.5	Cornell Univ.	61.09
SUNY, Stony Brook	47.5	Harvard Univ.	64
Univ. North Carolina	44.5	Johns Hopkins Univ.	64.5
Ohio State Univ.	46	MIT	59
Pennsylvania State Univ.	41.65	Univ. Pennsylvania	59
Rutgers Univ.	57	Princeton Univ.	59
Univ. Washington	48.5	Stanford Univ.	56
Univ. Wisconsin, Madison	44	Yale Univ.	65

Source: Council on Government Relations, data for 1996–97. CALTECH, California Institute of Technology; SUNY, State University of New York; MIT, Massachusetts Institute of Technology.

two federal 'cognizant agencies' (the Department of Health and Human Services, and the Office of Naval Research in the Department of Defense) saying how much they think they will need to cover the indirect costs of their research portfolio for the next few years. A set of strict rules is published by the Office of Management and Budget (see, for example, <http://www.whitehouse.gov/WH/EOP/OMB/html/circulars/a021/a021.html>).

Allowable indirect costs include provision of library facilities, supply of utilities to laboratories, and support to researchers from grant applications. Not allowable are advertising and public relations costs, contributions and donations, entertainment, fines and penalties, lobbying costs and the costs of defending fraud proceedings. 'Pre-grant' institutional costs of starting new projects, or of closing down old ones, are also not allowable, although they can be significant.

The relevant federal agency examines a university's proposals in detail to see what proportion of costs has been allocated to research. A process of negotiation follows, resulting in an average 'indirect-cost rate' for the university. This is the ratio, for the sponsored research effort across the university as a whole, of the allowable indirect costs to the modified total direct costs. (The latter costs are all attributable to individual projects, excluding the costs of large capital expenditure and subcontracts above certain thresholds.) These modified costs are used to avoid obvious distortions from large items of capital expenditure.

Current UK practice is to calculate indirect costs only on the basis of salary direct costs. This provides an incentive for the inclusion of salary items in grants as this may be the only way a project can recover any of

its indirect costs. The US system avoids this type of problem.

Negotiated indirect-cost rates vary across the United States from less than 40 to more than 70 per cent (see Table 1 for a selection of rates, and Table 2 for an example of the breakdown of indirect costs in one institution). There are good reasons for this variation among institutions. First, research is more expensive in some subjects than in others. Second, there are marked regional differences. Energy costs at different times of year can vary dramatically between, say, Phoenix, Arizona, and Chicago, for example. Third, and perhaps most important, there are different incentives to private and public universities to negotiate on their rates — public universities have an income stream from state governments, which can in some cases be used to cover some kinds of indirect costs; private universities do not.

The first two columns in Fig. 1 show the average differences between the indirect-cost rates proposed by US universities and the rates finally agreed, and also how these rates differ between private and public universities.

The indirect-cost rate agreed between the university and the cognizant agency is applied to every federal government grant and contract for research at that university (at least in theory — see below), and is the starting point for the university in negotiating a rate for indirect costs with other project funders. The same rate is used for all faculties across the university (although in some cases a separate rate is calculated for, say, the medical school or off-campus laboratories).

Teaching or research?

Why is it not possible to specify exactly the indirect costs of research in each university

simply by referring to the rule book? A primary difficulty is that, much as funders and administrators would like to tease them apart, teaching and research are intertwined. For example, an undergraduate or postgraduate helpfully engaged in a project is both a teaching cost and a research benefit. How is the principal investigator's time spent in this case divided between teaching and research? How is these apprentices' use of the library to be divided between their being taught or their conducting research? To ask these questions is to see that they are ultimately silly. Nevertheless, rough and common-sense answers are ineluctable parts of any estimate of indirect-cost rates. Even in the United States, with its strict set of rules, the process is still ultimately a subjective matter, and resolving the debate about the proportion of a particular laboratory's time that should be allocated to research is a non-trivial, even protean, task.

A similar calculation is carried out to identify the indirect costs associated with teaching, which can be directly relevant for certain externally funded courses. Typically, this rate is significantly higher than the rate for research, owing to the range of student services offered which are in most cases 100 per cent attributable to instruction, and because a high proportion of library services are attributed to instruction (perhaps not adequately reflecting the relative costs of supporting the research side of a library).

US universities have to devote time and money to keeping track of the relative proportions of time spent on research and teaching by individual faculty members, together with a multitude of other items which can affect the attribution of indirect costs to externally sponsored research. No figures are available for these administrative costs, which are not easy to estimate. We guess that they account for about one or two (possibly more) of the 50–60 percentage points in the agreed indirect-cost rates in Table 1.

The offsetting benefit is that this encourages a full understanding of the costs associated with different elements of the work

being done, both by the central administration and by the faculty. And, perhaps more important from the point of view of funders, it ensures that the same indirect cost is not accounted for twice — once for research and once for teaching. But all this has its own substantial costs, not only in administrative procedures but also, sadly often, in friction between faculty, university administration and government.

There are useful lessons from the virtues and the vices of the US system to be learned by the United Kingdom. Transparency encourages clear thought about the costs and benefits of particular indirect-cost items (in particular, it usually shows that — contrary to some scientists' suspicions — their colleagues in the humanities are not being subsidized by the indirect costs on science grants).

But all this costs time, money and emotion between researchers and administrators. Well handled, the clarity and accountability of the US procedures have benefits for both the university and the funders. At the same time, care is needed not to overdo the accounting and reporting requirements on universities. After all, the ultimate purpose of the exercise is to deliver quality research and teaching.

Indirect costs are real costs

For the holder of a hard-won research grant (often funded below the level requested), there can be a natural, if unreflective, tendency to see indirect costs as money wasted. In the United States, this is stereotypically the line taken by both researchers and funders. If only this money was not being 'siphoned off' by the administrators, the argument runs, there would be even more to fund research. This common misapprehension was one of the reasons why the term 'indirect costs' was replaced by 'facilities and administration costs' in the early 1990s. It is doubtful whether changing the name has changed the argument.

Such failure to recognize that indirect costs are real costs has underlain congressional pressure to reduce indirect-cost rates

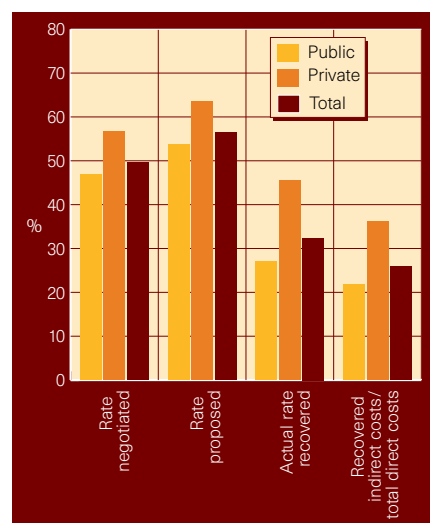


Figure 1 Average indirect-cost rates for US universities (Source: Council on Government Relations).

over the past decade or so. There have been significant changes since the high-profile case in which Stanford University was criticized for allegedly including illegitimate items in the overheads covered by federal funds. There has been a tightening of what is included in the Office of Management and Budget rules: the amount that can be claimed for administration in the indirect-cost rate has been capped at 26 per cent; and more stringent accounting requirements on universities have been introduced.

Of course it is important to have pressure on universities to operate as efficiently as possible so that indirect costs are at the necessary minimum. But indirect costs are very real costs for universities. The US system exposes the inherent tensions: funders and researchers have a short-term interest (for different reasons) in driving down the fraction of their grant spent on indirect costs, leaving a larger fraction for the more visible direct costs. And this view often attracts political support. On the other hand, administrators seek to maximize indirect costs. This enables them to provide the vital infrastructure for research, and also brings in money to allow the university flexibility in starting new projects and winding down old ones.

This tension among researchers, administrators and funders is healthy if moderated by proper understanding of the issues. In the United Kingdom, where there is less history of debate of these questions, some funders are emphasizing direct costs, and university administrators are making the case for indirect costs, but the researchers are comparatively silent.

Cost sharing

Although the principle underlying the funding of US research remains full-cost reimbursement, it is not at all clear that this is

Type of cost	Proportion of modified total direct costs (per cent)
Administration (total)	26.0
General administration and services	7.1
Departmental administration	15.7
Sponsored projects	1.8
Other administration	1.4
Facilities (total)	33.0
Depreciation of buildings	4.7
Depreciation of equipment	2.9
Library	4.0
Interest	2.2
Plant operations and maintenance	19.2
Overall indirect-cost rate	59.0

Source: Princeton University.

being delivered in practice. There often seems to be an implicit belief by some funders, in both federal and private sectors, that universities have a responsibility to cover some research costs from 'their own' funds.

Universities use the federal rate as a starting point for negotiation with non-federal funders (such as industry or charities) but often agree to carry out the work for less (the risk, of course, is to lose the work to someone else). And even federally funded projects do not always bring with them the full amount for indirect costs.

When it comes to the funding of individual projects, there is a strong temptation for funding agencies to argue down the proportion of money directed to indirect costs, so allowing more projects to be funded. At least superficially, this makes good sense from the perspective of the funding agency and the faculty researcher. But, in the long run, the underfunding of infrastructure will eat into the capabilities of the research enterprise.

The difference between what US universities should theoretically receive (if everyone reimbursed indirect costs at the full negotiated rate) and what they actually receive is demonstrated by columns 2 and 3 in Fig. 1. Across all universities, the figure suggests that, on average, indirect costs are recovered (from all funding sources) at the rate of 32 per cent, roughly two-thirds the average negotiated rate (for federally funded work) of 50 per cent. Again, the rate at which indirect costs are recovered varies between private and public universities, and it is notable that private universities recover a greater proportion of theoretical indirect costs than public ones.

It is not for us to decide whether US universities should be expected to make a contribution to the costs of externally commissioned research. But at present they clearly are doing so. And these costs have to come from somewhere. Such questions cry out for discussion of what fraction, if any, of the full costs of sponsored research should be the university's — rather than the external funder's — responsibility. The US Office of Science and Technology Policy is reviewing this issue. A report is scheduled to appear soon, and its conclusions deserve to attract wide interest.

Complex as it is, the US system does not require universities to demonstrate that money allocated for research overheads was spent directly, and in proportion, on the departments and groups that brought the money in. Nor should it. Having agreed an appropriate indirect-cost rate, the government leaves the universities to spend these infrastructure funds as they see fit. The transparent accounting systems in US universities, designed to track expenditure, are useful both in faculty debate and during subsequent rounds of indirect-cost negotia-

Comparing US and UK costs

The first difficulty in any comparison between US and UK research council indirect-cost rates is that the US rates in Table 1 and Fig. 1 are based on modified total direct costs (MTDC), whereas UK rates are generally based on direct-cost salary items. Any comparison would need a rough estimate of what fraction of MTDC is represented by salaries. Using a very rough guess of around two-thirds would translate the average US rate of 50–60 per cent in Fig. 1 to a UK rate of 75–90 per cent.

A second difficulty is that UK research councils allow as direct costs items which in the United States tend to be accounted as indirect costs (secretarial help, certain office expenses, and so on). If a fraction, f , of the UK direct costs are of this kind, we must move these costs from the denominator to the numerator of the indirect-cost

rate to compare with US rates. This reduces the UK rate that is effectively equivalent to US rates to around $[(75 \div 0)(1 - f) - 100f]$ per cent.

Third, the US indirect-cost rate includes components for buildings, generic and/or shared equipment and its depreciation, central computing facilities, and other things (see Table 2) that would fall in varying degrees into the higher education funding councils' (HEFC) research infrastructure costs in the United Kingdom, rather than being part of the research councils' indirect-cost rate. Under this 'dual support system', indirect costs in the United Kingdom are reimbursed by two different mechanisms: HEFC research infrastructure funds and research councils' indirect costs. If the numbers in Table 2 for Princeton University were taken as typical — admittedly an unreliable supposition

— we see them as falling roughly half to two-thirds in the research council indirect-cost category, and roughly half to one-third in the HEFC research infrastructure category (most, but not all, administration in the former; much of facilities in the latter).

Such a very rough guesstimate would suggest a UK indirect-cost rate in the neighbourhood of half to two-thirds of the 75–90 per cent of salary direct costs guessed above, which is to say very roughly in the range 37–60 per cent. To the extent that f is not zero, these figures will be somewhat smaller. For example, if f is around 0.05 (5 per cent of direct costs being for items that count as indirect costs in the US system), then our guesstimate of the US equivalent for UK research council indirect-cost rates falls to roughly 33–54 per cent.

tions. They also serve as evidence of good stewardship of public funds.

Conclusions

We believe that the openness of the US system has gone a long way to ensuring that all involved — researchers, administrators and funders — understand the issues. The system has benefited from it, in terms of efficiency and value for money, and in fostering a common understanding of aims and purposes. In the United Kingdom, because of the conditions attached to the new money awarded by the government, those involved have to get to grips with indirect costs.

Many universities in the United Kingdom have developed or are developing systems for greater accountability. The Committee of Vice-Chancellors and Principals commissioned a study last year to estimate the indirect costs of research in the UK system. The unpublished report leaves many of the key questions unanswered, not least because it does not distinguish between teaching and research — mainly because of lack of relevant information. Owing to this, and many other difficulties in the calculation, it is difficult to draw many useful conclusions from the study.

There are, of course, many differences — accounting, cultural and institutional —

between the UK and US higher-education sectors, so any comparison between indirect-cost rates in the two countries can be misleading. Nevertheless, we outline in the box above how we think the average US indirect cost rate of 50–60 per cent in Fig. 1 would translate into an equivalent UK rate on research council grants, as calculated by current UK rules, very roughly in the range 37–60 per cent. The current UK research council rate of 46 per cent happens to lie around the geometric mid-point of this range. Of course, this estimate is very rough, so this agreement may be coincidental.

Fig. 1 also shows that the average ratio of indirect costs actually recovered by universities in the United States is around 25 per cent of the total direct costs of the projects. The challenge for the UK research community, in return for the new injection of government money, is to understand and agree the equivalent UK figure. Those in charge of managing research can then deliver the right money to the right people in a transparent fashion without introducing unnecessary bureaucracy. □

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Micro-printing with crystal inks

Lia Addadi and Steve Weiner

Patterning a surface is the key to growing crystals on some areas of a substrate and not others. This can now be done, bringing the prospect of crystalline materials, patterned on nanometre scales, a step closer to reality.

Sophisticated new electronic or ceramic materials could be produced if we could grow arrays of crystals with a certain orientation and size, and at specific locations. This is evidently possible, even down to nanometre resolution. Certain organisms have evolved the ability to produce such crystal arrays, and with great fidelity. Materials scientists, often inspired by biology, have spent much time and effort in trying to emulate nature in this way. The report on page 495 of this issue¹ by Aizenberg, Black and Whitesides represents one of the best, if not the best, attempts yet.

In essence, Aizenberg and her colleagues

fabricated a surface with a series of raised topographic features, ranging in size from 3 to 50 μm , separated from each other by up to 100 μm . This 'stamp' was then placed in contact with different 'inks' — self-assembling molecules, with various charged or polar functional groups, that bound to the raised features. The inked stamps were then transferred to surfaces of silicon coated with metal, and the intervening areas were filled up with methyl-terminated molecules. The patterned surface was then suspended in a solution saturated with calcium carbonate, so that crystals could grow (see Fig. 1 of the paper on page 495 for details of the experi-

mental procedure). Calcite crystals nucleated exclusively on the functionalized areas, even though under the same conditions they also nucleated on a surface composed entirely of the methyl-terminated substrate. Clearly it is the patterned distribution of substrates that is the key to achieving almost total accuracy with respect to the places where crystals are allowed or not allowed to nucleate.

Furthermore, by varying the nature of the functional groups, the metal coating, and the density, shapes and sizes of the raised features of the stamp, Aizenberg *et al.* could control the number of crystals that nucleated at a given site, the distance between sites and the orientations of the crystals themselves. In short, they have command of almost every aspect required for controlling crystal nucleation down to submicrometre resolution.

These elegant experiments have their conceptual roots in two powerful technologies — sophisticated soft-lithographic micro-contact 'printing' using self-assembled monolayers, and crystal growth under controlled conditions on designed substrates. The recent history of the latter field goes back to the mid-1980s, when Landau, Lahav, Leiserowitz and co-workers² grew crystals of glycine and sodium chloride under monolayer surfaces at the air–water interface, demonstrating that the structure of the monolayer directly controls the crystal plane from which nucleation occurs. This strategy was also used to study controlled nucleation of calcium carbonate³. Subsequent research probed further into the details, one study showing that the nucleated crystals could even be oriented in three dimensions with respect to the structure of the monolayer substrate⁴.

In 1993 came Kumar and Whitesides' demonstration⁵ that monolayers self-assembled onto a substrate can be employed for microcontact printing, and since then progress has been rapid⁶. The two fields merged with the successful use of lithographic and self-assembling monolayer techniques⁷ to produce a patterned surface onto which crystals of iron oxide were grown; the resolution of the pattern was just under 10 μm , but the crystals were not orientated.

The main achievement of Aizenberg *et al.*¹ is the fidelity of their system. Almost 100% of the crystals nucleate at the predetermined nucleation sites, and with preferred orientation. The reason is that, once the crystals nucleate at these sites, diffusion of ions towards the crystals lowers the saturation state of the solution in the immediate environment around the nucleation sites, so preventing crystal growth elsewhere (Fig. 1). Aizenberg *et al.* also show that small changes in the structure of the functional molecules, or substituting one metal coat for another, results in the crystals nucleating from

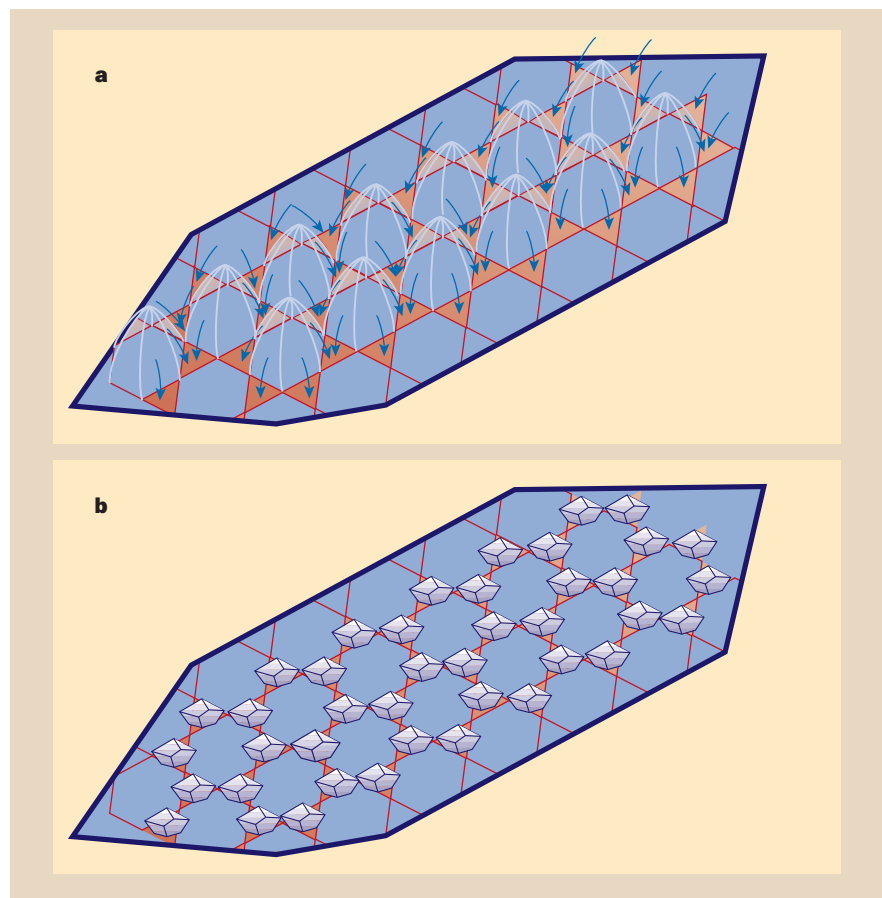


Figure 1 Controlling crystal growth — the principles behind the experiment of Aizenberg *et al.*¹. a, The patterned surface sets up zones of undersaturation (depicted under the domes), and areas of saturation (between the domes). Functionalized areas are depicted in red; methyl-terminated areas in light blue. The arrows show the flux of ions to the functionalized areas. b, The result of this process is oriented crystals that nucleate on the functionalized areas only.

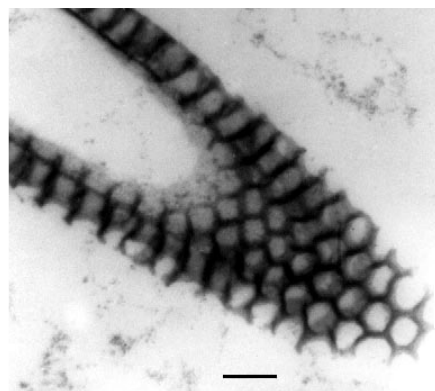


Figure 2 Transmission electron micrograph of part of the bacterium *Leptothrix*, showing the patterned mineralized coat composed of amorphous iron hydroxide¹⁰. (Photograph by Z. Mason, reproduced from ref. 11; scale bar is 200 nm.)

different crystal planes, and hence in their having different orientations. The number of crystals that nucleate at a site can also be controlled.

The next challenge is to grow the crystals in such a way as to control their shape and to merge them into a ceramic-like continuum. A step in that direction was taken by Xu *et al.*⁸, who, again taking a cue from biology, grew an array of calcite crystals under a monolayer by first forming a continuous precursor phase of amorphous calcium carbonate. Another aim is to achieve nanometre-scale patterning. Douglas and Clark⁹ used a biologically produced nanoscale template, namely the crystalline proteinaceous surface layer (or S-layer) of certain bacteria, for lithography. Individual protein complexes of ferritin were then selectively self-assembled onto the metal-coated template. Some bacteria, not surprisingly, can do it all by themselves with a precision of a few nanometres (Fig. 2).

The ultimate goal of many studies in this field is to produce nanometre-scale patterned crystalline materials for semiconductors, sophisticated ceramics, electro-optic materials and so on. The advances made over the past few years leave little doubt that this goal will, at least in part, be reached. Another benefit is that these studies are pointing the way to a better understanding of the biological processes that often inspired them in the first place. The experiments of Aizenberg *et al.*¹, for example, highlight the importance of directed diffusion at interfaces — a parameter that has not been sufficiently appreciated in work on the mechanisms of controlled biological crystal formation.

One day, diffusion control may even help solve health problems. Calcified crystal deposits almost inevitably form on the surfaces of artificial replacement materials implanted in the human body. By deliberately encouraging deposition of crystals on parts of these materials where they do no

harm, it might be that the working surfaces could be kept crystal-free and in good operational order. □

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Neurobiology

Slit, the midline repellent

W. A. Harris and C. E. Holt

In bilaterally symmetric animals, communication between the left and right halves of the body is mediated by neurons that send axons across the midline of the central nervous system (CNS). These axons move along seams, or 'commissures', that are thought to be evolutionarily ancient¹ — even very simple animals contract muscles on one side of their body to avoid a stimulus on the other side. Five reports in *Cell*^{2–5} and *Neuron*⁶ now describe the product of a previously known gene, *slit*, which is thought to be important not only in the formation of these commissures, but also in axon guidance and the migration of muscle cells.

Not all neurons are commissural, and there are many uncrossed projections in the CNS. Understanding what makes only some axons cross the midline has been a challenge. Several years ago, Marc Tessier-Lavigne and colleagues identified an axon-guidance molecule called netrin, which is secreted at the ventral midline of the developing vertebrate spinal cord. Axons from commissural neurons in the spinal cord are attracted to netrin⁷. At about the same time, Corey Goodman and colleagues identified two *Drosophila* mutants with midline-crossing defects. Whereas in *commissureless (comm)* mutants axons do not cross the midline at all, in *roundabout (robo)* mutants they relentlessly cross and recross it⁸.

Analysis of the *robo* gene and mutant phenotype suggested that it codes for a receptor to a midline repellent. Robo protein is expressed at high levels on the tips of growing axons that do not cross the midline⁹. Growth cones that cross the midline, by contrast, express very low amounts of Robo. Comm protein, which is expressed by midline glial cells, is involved in regulating Robo¹⁰. Comm is transferred to commissural axons when they reach the midline, causing downregulation of Robo. With Robo low, these axons cannot sense the midline repellent, so they cross. But as they cross, they upregulate Robo, so when they reach the other side levels of Robo are high and they cannot cross again. Thus, commissural axons cross only once because of their changing sensitivity to this

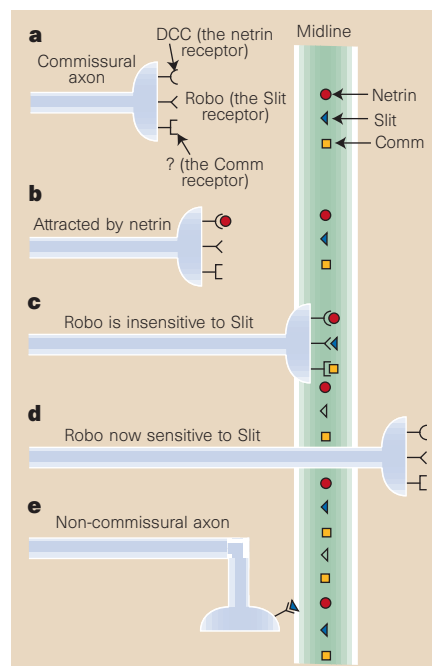


Figure 1 The function of Slit in axon guidance. a, Commissural axons express receptors for netrin, Slit and Comm. b, The commissural axon is attracted to the midline by netrin. c, Comm downregulates the Slit receptor, Robo, so the axon is insensitive to Slit and can cross the midline. d, Having crossed the midline the axon re-expresses Robo, making it sensitive to Slit and preventing it from recrossing the midline. e, Non-commissural axons are repelled by Slit, so cannot cross the midline.

mysterious midline repellent.

The five papers^{2–6} now identify the repellent as the product of the *slit* gene. First described in 1984 as an embryonic mutant by Nüsslein-Vollhard and Wieschaus¹¹, *slit* is one of many genes that affect the larval cuticle pattern in *Drosophila*. In 1988, Rothberg *et al.*¹² identified Slit as a secreted molecule expressed in midline glial cells, and showed that *slit* mutants have a collapsed axonal scaffold at the midline. Just as commissural axons bind Comm as they cross the midline, they also bind Slit¹³. So Slit is a good candidate for a Robo ligand (Fig. 1).

Kidd *et al.*³ show genetic interactions between *slit* and *robo* mutants, suggesting that these two molecules act in the same pathway. Brose *et al.*⁴ and Li *et al.*⁵ show that Slit binds to Robo on cell surfaces and in solution. There are at least three forms of Slit in mammals¹⁴, and at least two of Robo, all of which bind to each other across species boundaries. Moreover, human Slit repels rat motor axons. This is all excellent evidence that Slit is an evolutionarily conserved repulsive ligand for Robo. Interestingly, Slit also binds netrin and laminin *in vitro*, although we don't yet know why.

It is not uncommon for axon-guidance molecules to be used at more than one place in the brain. For example, as well as acting at the midline, netrin is expressed in the vertebrate visual system and in the body-wall muscles of flies. So, the finding of Ba-Charvet *et al.*⁶ and Li *et al.*⁵ — that the Robo–Slit system is used at other places in the CNS — is not surprising. The first clear case of axons being repelled by a diffusible ligand was in 1993. Adrian Pini¹⁵ showed that cultured axons from the olfactory tract are repelled from a region of the forebrain called the septum. Olfactory-bulb axons, it turns out, express high levels of Robo-2, whereas Slit-2 is highly expressed in the septum. Then there is the hippocampus. Hippocampal axons, which express Robo, do not invade the adjacent entorhinal cortex, which expresses Slit-2. Cell lines expressing Slit-2 can repel both olfactory-tract and hippocampal axons *in vitro*.

Although the new papers show that Slit should join the growing family of evolutionarily conserved, repulsive guidance factors in the CNS, Wang *et al.*² report the identification of Slit through a different approach — one that suggests a distinct function for Slit. During development, the axons of pain and temperature receptors enter the spinal cord and travel up and down on the same side for a short distance. They then produce branches along these axon shafts. The branches make synapses with the commissural interneurons that take the message of pain or temperature to the brain. By culturing these pain- and temperature-sensitive neurons in isolation, while exposing them to different CNS fractions, Wang *et al.* discovered that the fraction containing Slit dramatically promoted axonal branching and growth. So, although it has just been identified as a repellent, Slit can also serve as a positive growth- and branch-promoting substance.

The new studies raise further questions. For instance, is the branch-promoting activity of Slit on sensory neurons mediated through Robo? And why does Slit, the repellent, bind netrin, the attractant? Whatever the answers, by uncovering the repellent that keeps some axons from crossing the midline and others from recrossing, these studies shed considerable light on the ancient

mysteries of commissure formation. □

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Motor proteins

Another step ahead for myosin

Malcolm Irving and Yale E. Goldman

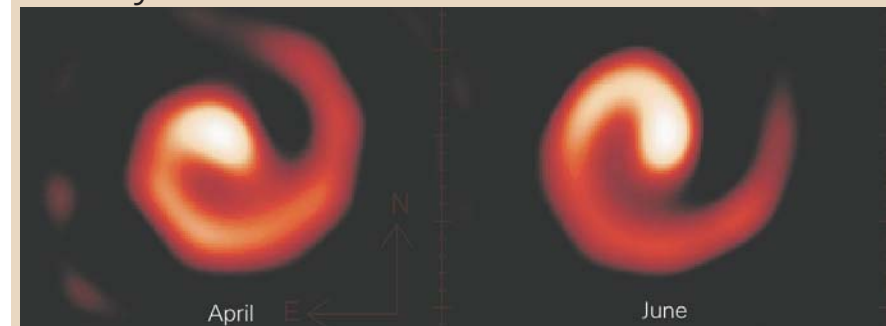
Eukaryotic cells contain many different protein motors, which use metabolic energy to transport cell components along polymer tracks such as actin filaments, microtubules or DNA. A single motor molecule moves along an isolated track in nanometre-scale steps corresponding to hydrolysis of single ATP molecules^{1–4}. But two studies on myosin — the motor protein in muscle — by Veigel *et al.*⁵ (page 530 of this issue) and Kitamura *et al.*⁶, show that each interaction with actin can include two or more sub-steps per ATP hydrolysed.

The head region of myosin, which embodies its motor function, contains a catalytic domain that binds actin and ATP, and an elongated carboxy-terminal domain containing a variable number of calmodulin-like light chains. The light-chain domain is thought to act as a lever arm in the motor mechanism^{7,8}. It is often connected to its cargo (which may be a vesicle or filament) through a coiled-coil tail.

Veigel *et al.*⁵ exploited the slow kinetics of two single-headed myosins, myr-1 from rat liver and brush-border myosin-I (BBM-I)

Astronomy

A dusty revolution



Collectors of unusual astronomical objects have another to add to their list: the first spiral star ever observed. Elsewhere in this issue (*Nature* 398, 487–489; 1999) Peter Tuthill and colleagues report high-resolution infrared images of a spiral structure in the hot dust around a Wolf–Rayet star (WR104). They use a powerful aperture-masking technique at the 10-m Keck telescope in Hawaii to produce images much better even than those taken by the Hubble Space Telescope.

Wolf–Rayet stars are a phase in the life of exceptionally hot, massive stars, just before they are thought to become supernovae. Some Wolf–Rayet stars are surrounded by shells of dust, but it has been a mystery as to how dust survives the harsh ultraviolet radiation they emit.

Now, not only have Tuthill *et al.* detected a spiral pinwheel in the dust around WR104, but they also watched it rotate every 220 days. The image above shows the dusty spiral as seen in April and June 1998. The authors say the spiral and its rotation are the consequence of a companion star. In their hypothesis, dust is created around the binary star where the stellar winds collide, and is then carried along with the orbital motion.

Whether every dusty star has a binary remains open for debate. But in this case, the images of the spiral are so good that the orbital period, distance and separation of the binary system can be inferred from its effect on the stellar dust, without ever detecting the two central stars.

Sarah Tomlin

from chicken intestinal epithelium, to reveal the substructure of the interactions between each of these motors and single actin filaments. The authors suspended a filament between two 1- μm plastic beads held by optical tweezers, then brought this into contact with a myosin molecule bound to nitrocellulose. They detected transient interactions between the myosin head and actin by the associated reduction in thermal fluctuations of the beads.

Veigel and colleagues found that the filament is displaced by about 6 nm at the start of each interaction, then, surprisingly, by another 5.5 nm 100–300 ms later. The

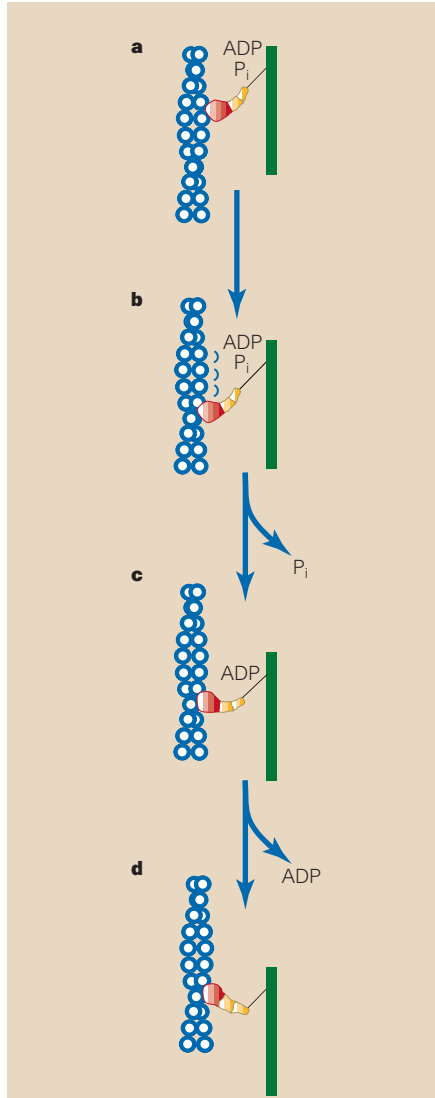


Figure 1 Different ways in which a myosin S1 head might produce sub-steps as it moves along an actin filament. The catalytic domain is shown in red, with the light chains in yellow. Actin is shown in blue, with the barbed end downwards. The results of Kitamura *et al.*⁶ indicate that S1 might jump between actin-binding sites (a $\rightarrow$ b). Alternatively, or additionally, according to the results of Veigel *et al.*⁵, sequential conformational changes in the myosin head might be linked to the release of inorganic phosphate (P_i) and ADP (b $\rightarrow$ c $\rightarrow$ d).

interval between the two sub-steps did not depend on the concentration of ATP. However, the interval between the second sub-step and the end of the interaction was shorter at higher concentrations of ATP — as expected if each interaction is terminated by the binding of ATP to myosin. With skeletal-muscle myosin heads (termed S1), only one 5.5-nm step was observed, within 5 ms of the start of the interaction.

The authors suggest that the sub-steps seen with myr-1 or BBM-I are coupled to two intermediate steps in the ATP hydrolysis cycle. These steps could be the release of inorganic phosphate (P_i) from the active site (Fig. 1; b $\rightarrow$ c), followed by the release of ADP (Fig. 1; c $\rightarrow$ d). This idea fits with image reconstructions of BBM-I heads bound to actin filaments⁹. When ADP dissociates, the light-chain domain of BBM-I tilts by about 6 nm, similar to the sub-steps seen by Veigel *et al.*⁵ for this motor. Moreover, S1 from skeletal muscle does not tilt in response to ADP (ref. 10; R. Diaz and R. A. Milligan, personal communication), supporting the identification, by Veigel *et al.*, of BBM-I's second sub-step with ADP release.

However, Kitamura *et al.*⁶ have reported multiple sub-steps for skeletal muscle S1. These authors captured S1 on the tip of a scanning-probe microscope, then moved it close to a bundle of actin filaments in the presence of ATP. Interactions between the myosin and actin could again be detected by decreased thermal fluctuations. A series of up to five sub-steps of about 5.3 nm each, usually all in the same direction, was observed at the start of each interaction. Although the duration of a single interaction varied inversely with ATP concentration, the interval between the sub-steps, only about 4 ms, did not depend on the concentration of ATP. This is strong evidence that an interaction containing several sub-steps requires only a single molecule of ATP.

Although the two papers^{5,6} report sub-steps of the same amplitude, the multiple sub-steps observed for S1 by Kitamura *et al.*, but not by Veigel and colleagues, produce a large discrepancy in the total displacement per interaction. This discrepancy is the latest episode in a long-running controversy. The divergent results probably reflect differences in the techniques used by the two groups: the methods of attaching actin and myosin to the transducers; the geometrical relationships between the two proteins; and the mechanical compliance and dynamic response of the experimental systems. For example, actin filaments slide over an S1-coated surface at least three times faster with the biotin/avidin attachment method used by Kitamura *et al.* than with the nitrocellulose method used by Veigel *et al.*, and the faster velocity is close to that seen with the native protein¹¹. Another possibility is that the high stiffness perpendicular to the filament of the scanning probe

used by Kitamura *et al.* suppresses the dissociation of myosin from actin.

What can the new results tell us about the mechanism of the myosin motor? The isoform differences probably reflect functional specializations — BBM-I and myr-1 may control vesicle transport and the viscoelasticity of the cell cortex, whereas skeletal-muscle S1 must work with hundreds of partners to produce rapid filament sliding. These characteristics may require differences in the step size, the fraction of time spent attached to actin, or the relationship between ADP release and filament sliding¹².

Because the S1 lever arm is about 9 nm long, and the BBM-I and myr-1 arms are even longer, the presence of one or two 5–6-nm sub-steps is consistent with the tilting lever-arm model (Fig. 1; b $\rightarrow$ d). But this model cannot readily explain the total displacements of almost 30 nm sometimes seen by Kitamura *et al.* for S1, or the uniform 5.3-nm sub-steps that they report. These sub-steps are roughly equal to the 5.5-nm separation of actin monomers along each strand of the actin filament, suggesting that myosin heads can jump repeatedly between actin-binding sites during a single ATPase cycle (Fig. 1; a $\rightarrow$ b). A series of as many as five such jumps in the same direction, as observed by Kitamura *et al.*, would seem to require a mechanism with novel structural and physicochemical concepts.

Whatever the final interpretation of these new studies, they show that single-molecule techniques can reveal intermediate transitions in protein–protein interactions, and give us a first glimpse of mechanical substructure within single actin–myosin interactions. This is a big step towards understanding the mechanism of energy transduction by motor proteins. □

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Alzheimer's disease

In search of γ -secretase

John Hardy and Alain Israël

When the race was on to identify the genes on chromosomes 14 and 1 which, when they contain mutations, lead to early-onset Alzheimer's disease, it was generally thought that they would encode enzymes involved in the processing of the β -amyloid precursor protein (APP). It is a derivative of APP, the neurotoxic peptide A β , that is implicated as the main agent of the disease. When candidate genes, called the presenilins, were cloned, their only known protein homologues were apparently involved in vesicle transport or the Notch developmental pathway in the nematode *Caenorhabditis elegans*. Subsequent work in transgenic mice and transfected cells, however, soon showed that mutations in presenilins all altered APP processing and caused increased levels of the 42-amino-acid β -amyloid derivative A β 42. (See ref. 1 for references.)

Since then there has been intense interest in the relationship between A β production, the presenilins, the Notch pathway, vesicle transport and Alzheimer's disease. The latest findings appear in four papers in this issue²⁻⁵, with further results being reported at a Keystone meeting in March. These studies do not demonstrate that Notch signalling is directly relevant to Alzheimer's disease. But they do show that presenilins regulate both APP processing and Notch signalling by influencing unusual protein cleavage events.

Receptors of the Notch family mediate cell-cell interactions that specify cell fate during development. They are large, single-pass transmembrane proteins (Fig. 1) and their ligands are expressed at the surface of neighbouring cells. How Notch signalling results in transcriptional activation of its target genes remains a mystery, but one model has it that proteolytic cleavage of Notch results in release of the intracellular domain⁶⁻⁹, which translocates to the nucleus and associates with a DNA-binding subunit. A processing step responsible for releasing the intracellular domain takes place in or near the transmembrane domain⁶. So Notch undergoes proteolytic events that resemble those involved in cleavage of APP (where first α - or β -secretase, and subsequently γ -secretase activities, operate; Fig. 1).

Genetic evidence that presenilins are also involved in Notch signalling comes from both *C. elegans* and mice. In *C. elegans*, the presenilin gene *sel-12* facilitates the activity of the two Notch genes *lin-12* and *glp-1*, and reducing *sel-12* activity suppresses the effect of increased *lin-12* activity¹⁰. In mice, inactivation of presenilin-1 results in phenotypes

associated with reduced Notch activity¹¹. Finally, co-localization of Notch and presenilin in embryos of the fruitfly *Drosophila*¹², as well as a direct physical interaction between presenilins and Notch, has been observed in mammalian and *Drosophila* cells¹³. However, the exact role of presenilins in the Notch pathway has remained elusive, and these proteins have been tentatively attributed functions in either Notch processing directly, or in trafficking Notch to the correct compartment for that processing to occur.

The new work²⁻⁵ emphasizes the connection between Notch and presenilins, and indicates that presenilins are required for the production of Notch proteolytic products implicated in signalling. Ye and colleagues (page 525)², and Struhl and Greenwald (page 522)³ show that loss-of-function mutations in the *Drosophila* presenilin gene exhibit a lethal Notch-like phenotype. The amount and subcellular localization of Notch seems

to be identical in wild-type and mutant embryos, implying that the defects actually result from the absence of Notch signalling activity.

Ye and colleagues analyse the Notch species in wild-type and mutant embryos, and conclude that some proteolytic cleavage event giving rise to low-molecular-weight, Notch-derived fragments is inhibited in the absence of presenilin. Struhl and Greenwald reach a similar conclusion. They introduce a Notch-derived transgene into *Drosophila*, one engineered so that activation-associated processing followed by nuclear translocation of the intracellular part of the receptor can provide a direct colorimetric assay for signalling⁷; in the absence of presenilin, no nuclear Notch activity is observed.

De Strooper *et al.* (page 518)⁴ investigate the effect of presenilin on Notch processing by introducing a constitutively active form of murine Notch-1 (ΔE ; see Fig. 1) into fibroblasts derived from presenilin-1-knockout mice. This construct had been previously used to identify a proteolytic cleavage site located in or near the transmembrane region of Notch. This site is constitutively cleaved in the ΔE construct, and ligand-inducibly cleaved in the full-length

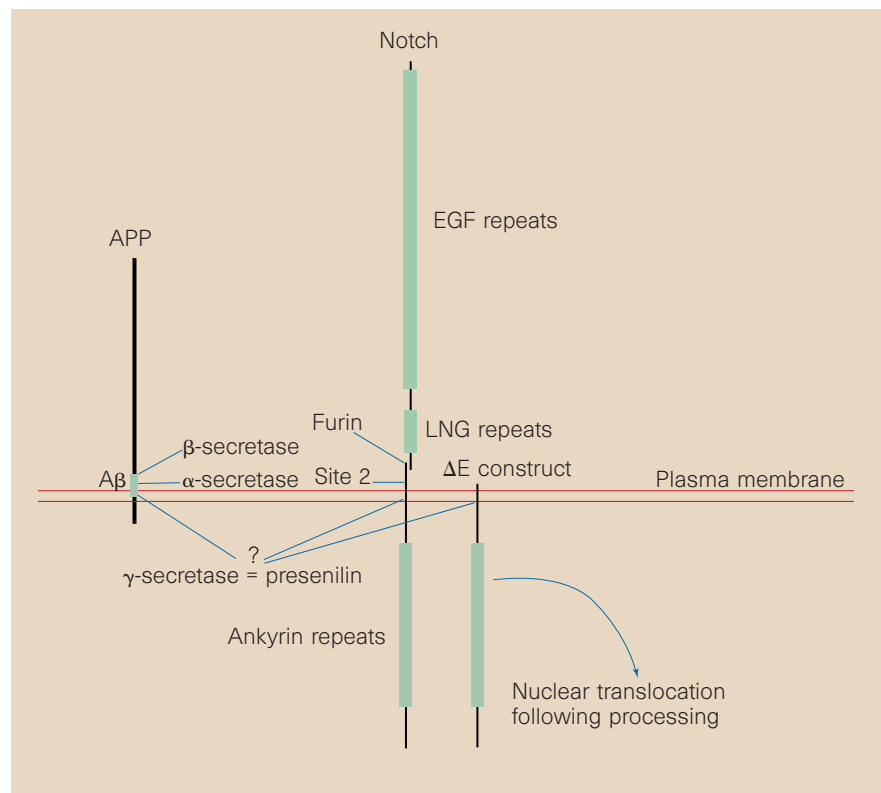


Figure 1 Proteolytic steps involved in the maturation of β -amyloid precursor protein (APP) and Notch. APP is cleaved at either of two extracellular sites by α - or β -secretase, followed by intramembrane cleavage by γ -secretase. Successive cleavages by β - and γ -secretases release the amyloid peptide (A β), which lies between these two cleavage sites. Notch is first cleaved by furin in the trans-Golgi network; the two resulting fragments remain associated at the plasma membrane. A putative ligand-induced second cleavage is indicated (site 2). The last cleavage⁶ releases the intracellular domain of the receptor, which translocates to the nucleus to activate transcription. The constitutively active ΔE construct discussed in the text is also shown. Three types of repeats present in the Notch molecules — epidermal growth factor (EGF), Lin-12/Notch/Glp-1 (LNG) and ankyrin — are indicated.

receptor, and mutation of the site reduces cleavage and abolishes signalling⁶. Interestingly, this processing does not take place in presenilin-1-knockout cells. In addition, the processing of the ΔE construct is inhibited by a γ -secretase inhibitor designed from the relevant APP sequence^{4,5}. Struhl and Greenwald similarly demonstrate that a ΔE -like construct is unable to signal in the absence of presenilin.

So these three groups²⁻⁴ all conclude that presenilin is required for release of the intracellular domain of Notch from the plasma membrane. Struhl and Greenwald, and De Strooper *et al.*, however, propose that presenilin-1 acts by facilitating the activity of the protease concerned or is the protease itself. This hypothesis is also adopted by Wolfe and colleagues (page 513)⁵, who suggest that presenilin-1 might be able to cleave both APP and Notch. Their work involved mutagenesis of two transmembrane aspartate residues in presenilin-1, which completely abolished APP cleavage. (At the Keystone meeting, C. Haass reported similar data from mutagenesis of presenilin-2; the relevant aspartate residues are conserved in all known presenilins.) Wolfe *et al.* propose that these aspartates are the active site of proteases that can cleave APP or Notch inside the membrane.

The results of the mutagenesis experiments are provocative, but they do not conclusively show whether the presenilins are important in trafficking or in cleavage. They could be causing defects upstream of the cleavage events, either by altering trafficking of the substrates (APP and Notch), or by altering trafficking or activation of the protease or proteases involved in γ -secretase-type cleavages. Indeed, alteration of substrate trafficking is the interpretation favoured by Ye and colleagues². They use a *Drosophila* Notch-derived construct similar to ΔE , which — contrary to the results of Struhl and Greenwald — they find can still signal in the absence of presenilin. So Ye *et al.* conclude that presenilin acts upstream of this processing step.

In this context, it is notable that, in humans, the apolipoprotein E genotype modulates the age of onset of Alzheimer's disease encoded by APP-717 mutations (which are believed specifically to affect γ -secretase cleavage) but not that of presenilin-encoded disease^{14,15}. From this it would seem that these events are genetically distinct, a conclusion supported by other lines of evidence. At Keystone, R. Nixon reported that presenilin and APP mutations have different effects on the vesicular trafficking of APP; the function of *spe-4*, the 'forgotten' presenilin, seems more closely connected to vesicle trafficking in *C. elegans* testes than to proteolysis¹⁶; and most (but not all) studies in mammalian cells indicate that presenilins are located in the endoplasmic reticulum or

in the Golgi, yet the processing events described above probably occur at the plasma membrane.

Nonetheless, the case is far from nailed either way. Both views remain viable — that presenilins are indeed γ -secretase, or that they instead directly traffic APP and Notch to the right cellular compartment for γ -secretase processing. Direct biochemical experiments will be required to distinguish between them.

Finally, a word of caution. Drugs targeting γ -secretase are seen as a possible treatment for Alzheimer's disease. Given the emerging role of Notch in the haematopoietic system¹⁷, however, and the firm implication that Notch and APP may be processed by the same cellular machinery, such drugs may have unwanted immunosuppressive effects. □

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Supercooled water

Going strong or falling apart?

Srikanth Sastry

For a tiny, almost spherical molecule made of just three atoms, water exhibits remarkably rich behaviour. In the crystalline state, water exists in at least 12, and possibly 14, forms of ice. Echoes of this polymorphism are to be found in the amorphous state as well. When prepared as an amorphous solid, or glass, by ultrafast cooling or by depositing vapour on a cold substrate, water is found in two forms: a high-density and a low-density form¹. Similarly, it has been proposed that liquid water exists in two corresponding forms at low temperatures, as a result of a novel liquid–liquid phase transition²; however, decisive evidence for this transition is still lacking.

Not only can water exist in two glassy forms, but also in its approach to the glassy state, it appears to exhibit two drastically different behaviours. On page 492 of this issue, Ito, Moynihan and Angell³ describe evidence that water changes character, from being a highly 'fragile' liquid at temperatures above 236 K to a 'strong' liquid near 136 K, its glass-transition temperature at atmospheric pressure (Fig. 1). They reach this conclusion by comparing kinetic and thermodynamic measures of a liquid's 'fragility', which are found to be consistent for all liquids they consider, except water.

In order to form a glass, a liquid has to be cooled below its freezing temperature, but without allowing crystallization to occur. A liquid 'supercooled' in this manner grows progressively more viscous. Viscosity controls how fast a liquid relaxes to equilibrium when it is perturbed; for example, when its

temperature is altered. When the viscosity grows sufficiently large, to 10^{13} Pa s, the liquid 'falls out of equilibrium' and is trapped into a solid, but microscopically disordered, state — the glass. Yet the high viscosity at the glass transition is reached differently by different liquids. When displayed on an Arrhenius plot

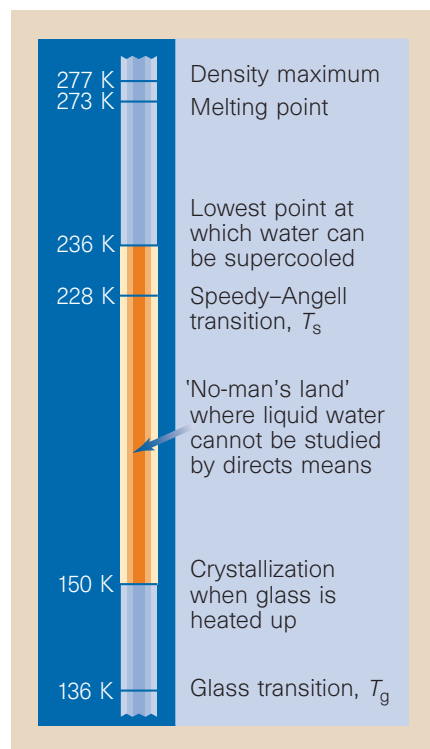


Figure 1 The mysterious properties of water below 0 °C (273 K). (Modified from ref. 15.)

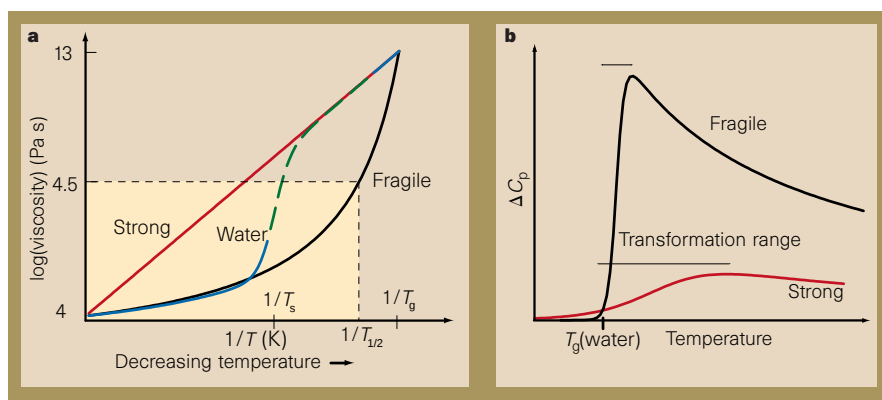


Figure 2 Kinetic fragility. a, The fragility plot. In this representation, the viscosity, $\eta(T)$, of 'strong' liquids shows a uniform increase, whereas for fragile liquids, the viscosity increases faster as the temperature gets lower. On the basis of available evidence, water changes from fragile to strong behaviour before the glass transition is reached, as shown schematically here. T_s marks the temperature where various properties of water appear to diverge with a power-law singularity. T_g is the glass transition temperature. $T_{1/2}$ is defined such that $\log(\eta(T)) = \frac{1}{2} \log(\eta(T_g))$. The kinetic fragility, $F_{1/2} = 2(T_g/T_{1/2}) - 1$, is closer to one the more fragile the liquid is. b, The excess specific heat of the liquid ΔC_p near the glass transition temperature T_g . Fragile liquids show a sharper drop than strong liquids because their viscosity increases more rapidly (over a narrower interval of temperature) to the value at T_g . The transformation range is the interval ΔT_g over which the specific heat drops to glass values, as indicated. Water exhibits behaviour illustrated here for strong liquids, but only up to 150 K where it crystallizes.

($\log(\text{viscosity})$ versus $1/T$; Fig. 2a), some liquids show a steady increase, but for others, the viscosity rises faster as temperature gets lower. The former are termed strong liquids, whereas the latter are fragile.

Fragility can be quantified in various ways; Ito *et al.*³ consider two kinetic measures, and demonstrate consistency between them. One measure of the kinetic fragility, $F_{1/2}$, relates to the temperature, $T_{1/2}$, at which $\log(\text{viscosity})$ reaches half its value at the

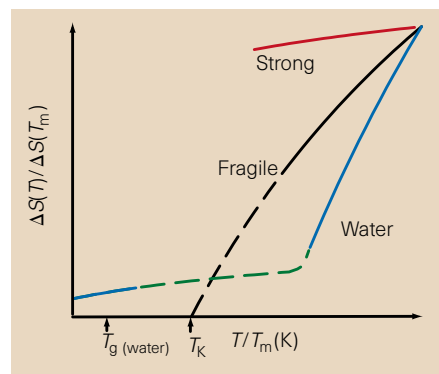


Figure 3 Thermodynamic fragility measured by Ito *et al.*³. The entropy difference between the liquid and the solid, $\Delta S = S(\text{liquid}) - S(\text{solid})$ normalized by its value ($\Delta S(T_m)$) at the melting temperature T_m , shows a stronger drop with temperature (T/T_m) for fragile liquids than strong liquids. Water, in this representation, is seen to be an extremely fragile liquid at high temperatures. The extension (dashed line) illustrates the expected behaviour of water's entropy in the range of 150 K to 236 K where it cannot be measured. T_K is the 'Kauzmann temperature' (illustrated for a fragile liquid) where, by extrapolation, ΔS goes to zero.

glass-transition temperature T_g (Fig. 2a). The other is the normalized width of the 'transformation range' over which glass formation occurs, $\Delta T_g/T_g$ (Fig. 2b). The specific heat drops from a high value for liquid to a low value for glass in this range, and corresponds to a rise in viscosity by two to three orders of magnitude (Fig. 2b). Ito *et al.* go on to show that these kinetic measures of fragility are also consistent with a thermodynamic measure they propose, which is based on how rapidly the entropy of the liquid drops below its value at the melting temperature, T_m . The more fragile liquids show a more rapid fall in entropy (Fig. 3). This is very gratifying, because the notion of fragility, and indeed the name, harks back to the Adam-Gibbs theory⁴ of the glass transition, according to which the increasingly rapid rise of viscosity on cooling is due to a progressive reduction in the liquid's entropy. It appears that all around, there is much consistency.

Water spoils this tidy picture. The transformation range for water proves to be quite large, and implies that near T_g water is a very strong liquid, much like silicon dioxide, a liquid that shares many similarities with water. On the basis of how rapidly its entropy changes near the melting temperature, however, water emerges as the most fragile of all liquids considered (Fig. 3).

Although puzzling at first sight, this observation is consistent with the striking behaviour of water near 228 K (T_s in Fig. 2a), first noted by Speedy and Angell⁵. They showed that many dynamic quantities (such as viscosity and relaxation times) and also thermodynamic ones (for instance, isother-



100 YEARS AGO

After a visit to Egypt in 1894, Miss Benson tells us she first entertained the idea of undertaking some excavation, and in the following year she obtained permission to clear away some of the earth that still covered the ruins of the temple of Mut. For three seasons Miss Benson and her friend, Miss Gourlay, have occupied themselves in removing debris and, though they have made no very startling discoveries, they have succeeded in correcting Mariette's plan of the temple in several details ... It will be seen, therefore, that Miss Benson and Miss Gourlay have had some reward for their three seasons' work; and, although surface-excavation at Karnak is not a very arduous or difficult undertaking, it is not unreasonable that they should be proud of having obtained the first permission to excavate given to women in Egypt.

Whether their example will be followed by other ladies remains to be seen, though we think on the whole such work is perhaps better left to the male professional digger, who can camp on the spot, and having knowledge of Arabic is naturally better able to control his men, and check to some extent the thefts of the smaller antiquities.

From *Nature* 6 April 1899.

50 YEARS AGO

A new 16-mm. sound and colour film, 'The Nature of Plastics', which has been sponsored by Bakelite, Ltd., was shown for the first time at the British Council Theatre in London on April 5. The film, which runs for about 20 minutes, is a scientific documentary and is designed to explain to the intelligent layman the broad principles of the structural characteristics of plastics and their associated physical properties. The molecular chain structure is particularly emphasized and is illustrated by large-scale molecular models. Then, with the aid of mechanical analogies, the packing of the chains is related to the physical properties, tight-, medium- and loose-packing corresponding to fibres, heat-softening plastics and rubbers respectively. In the case of the rubber structures, the chains can be locked by means of cross-connecting atoms, and this produces a heat- and chemical-resistant material which is called a heat-hardening or thermosetting plastic.

From *Nature* 9 April 1949.

mal compressibility) change with temperature in a power-law fashion (proportional to negative powers of $(T - T_g)$) and, by extrapolation, would become infinitely large (or diverge) at the 'singular' temperature T_g . Regardless of a genuine phase transition at T_g , these trends may signal dramatic qualitative changes in water's properties in this temperature range. On general theoretical grounds⁶, and also on the basis of constraints imposed by available measurements⁷, one expects the entropy to fall rapidly in this temperature region, and to stabilize at the smaller rate of change observed near the glass-transition temperature, $T_g = 136$ K (Fig. 3).

There is gathering evidence from computer simulations that the apparent divergences in the dynamic properties of supercooled water discussed by Speedy and Angell⁵ (and analogues at different pressures) are to be understood as realizations of behaviour predicted by mode-coupling theory⁸. In its simplified form⁹, mode-coupling theory predicts a dynamical transition temperature near which relaxation times change in a power-law fashion and at which they become infinite (that is, relaxation ceases to occur). A more detailed analysis reveals that the relaxation times do not in fact become infinite at the predicted temperature, so instead of describing the glass transition as originally thought, mode-coupling theory defines a 'crossover' temperature (which would be T_g for water at atmospheric pressure) that marks a change in the character of the liquid's dynamics.

At lower temperatures, significant energy barriers are expected to separate distinct arrangements of particles in the liquid, as originally discussed by Goldstein¹⁰, and impede structural rearrangements. The agents of structural relaxation in water near room temperature and atmospheric pressure are 'bifurcated bonds'¹¹ — defects in the hydrogen bond network — that provide a means of structural rearrangement at low energy cost. Computer simulations seem to indicate that the density of such defects approaches zero near T_g . At lower temperatures, the structure of the liquid would be that of a disordered tetrahedral network of hydrogen bonds, whose restructuring requires substantial expenditure of energy.

Many pieces of the puzzle are now in place, but gaps remain. The entropy of water near T_g appears to be too low compared with theoretical estimates of the entropy of a disordered tetrahedral network¹². Conversely, recent diffusivity measurements¹³ of water near T_g appear too high to be compatible with Adam-Gibbs theory, which relates the relaxation times of liquids to their entropy. The drastic changes in the behaviour of water near T_g raises the question of whether such changes are to be expected in other liquids as well. There is also evidence¹⁴ of devia-

tions from Adam-Gibbs predictions above T_g for a variety of liquids, that need to be explained. The biggest gap is perhaps a satisfactory theoretical justification of the Adam-Gibbs relationship, which has so far remained elusive. Exceptions are expected to illuminate, if not prove, the rule. Making sense of water's behaviour, it appears, will sharpen our understanding of supercooled liquids in general. □

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Cell biology

Boa constrictor or rattlesnake?

Tom Kirchhausen

During nervous transmission, neurotransmitter-filled vesicles fuse with the presynaptic membrane. Endocytosis — an uptake mechanism involving invagination of the cell membrane — then follows, returning the fused membrane to the interior of the cell to be recycled. The protein dynamin is found at the neck of each endocytic invagination, or pit, and is essential not only for recycling of synaptic vesicles, but for clathrin-dependent endocytosis from the plasma membrane of all cells.

The accepted role for dynamin is that it acts as a 'pinchase'^{1,2} — that is, it self-assembles into a ring around the neck of a budding pit, then changes shape in response to binding or hydrolysis of GTP. It was assumed that this coordinated change in the conformation of the dynamin ring leads to constriction of the neck, and scission. On page 481 of this issue, however, Sever *et al.*³ propose that dynamin is not a mechanochemical transducer or 'boa constrictor', but that, like all other known

GTPases, it acts as a switch, or 'rattlesnake', to regulate the endocytic step.

Dynamin is a relatively large protein made up of four subunits, each of which contains a GTPase module. It is unusual among GTPases because it has a low affinity for GTP compared with the small and the heterotrimeric GTPases, and because its intrinsic rate of GTP hydrolysis is high and dramatically increased by polymerization. Pure dynamin can spontaneously form rings and spirals, and it decorates microtubules and lipid vesicles with helices of similar dimensions. Any condition that leads to ring formation also stimulates dynamin's GTPase activity.

The accepted model for how dynamin works arose after a variety of experiments showed that, if dynamin function is inhibited, there is no vesicle scission. When GTP hydrolysis was blocked (using a non-hydrolysable analogue⁴ or mutations in the GTPase domain⁵), or when dynamin's ability to self-assemble on membranes was

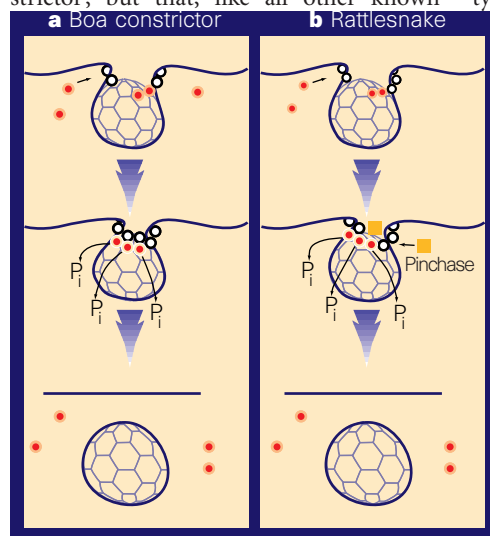


Figure 1 Two models for the action of dynamin. On clathrin assembly, the membrane deforms into buds. Dynamin tetramers (in the GDP-bound state; red) are recruited to the neck, which acts as a template for formation of the dynamin spiral. Bound GDP is exchanged for GTP, and on completion of a spiral around the neck of a bud — when dynamin tetramers at one end of the spiral stack on tetramers from the other — GTP hydrolysis is accelerated. a, The previous model suggested that dynamin acts like a 'boa constrictor' — GTP hydrolysis sends the conformational signal to proceed with scission. b, The results of Sever *et al.*³ indicate that dynamin could recruit 'pinchase', which carries out scission, to the neck.

inhibited⁶⁻⁸, endocytosis no longer occurred. Furthermore, if GTP was added to dynamin rings assembled around lipids, these lipid tubes fragmented into small vesicles⁹.

Sever and colleagues³ now show that inhibition of GTP hydrolysis can, in fact, increase the rate of endocytosis, raising significant questions about the original model. But what did these authors do that was different? From their earlier work, they knew that dynamin's carboxy-terminal region contains a GTPase effector domain (GED), which is distinct from the GTPase domain (located at the amino terminus). The GED is required to stimulate dynamin's GTPase activity when it forms rings — an effect that could be due either to an increase in the rate at which GDP is exchanged for GTP, or to activation of GTP hydrolysis.

Sever *et al.* first show that the GED acts as a GTPase activating protein (GAP), both when the GTPase domain is isolated in solution and in polymerized rings of intact dynamin. Then, guided by known mutants of rasGAP (a GAP for another GTPase, Ras), they created two dynamin mutants, dyn(K694A) and dyn(R725A). These mutant proteins turned out to have defects in the assembly-dependent GAP activity. Whereas dyn(R725A) directly affects the catalytic GAP function, dyn(K694A) partially interferes with the self-assembly of dynamin.

Because mutations in the GED impair dynamin assembly — and because GEDs associate with each other — we can assume that the GED domain forms part of the contact between stacked dynamin rings (forming the spirals that wind round on themselves). Presumably, dyn(K694A) allows the dynamin spirals to form, but with impaired communication between the rings, reducing stimulation of the GTPase activity. So these new dynamin mutants should be functionally equivalent to mild GTPase mutants, and would be expected to show impaired endocytosis. It was a surprise, then, that Sever *et al.*³ obtained exactly the opposite result. Overexpression of dyn(K694A) or dyn(R725A) leads to accelerated endocytosis of a probe molecule, transferrin, indicating that GTP hydrolysis, although required to complete the fission cycle, is not rate limiting for endocytosis.

So a new concept for dynamin function emerges (Fig. 1). GDP-dynamin is recruited to the forming bud, where it assembles into rings. Dynamin's intrinsic GAP is activated on completion of a spiral around the neck of the bud, when dynamin tetramers from one end of the spiral stack onto tetramers at the other end (rather than by lateral interactions along the dynamin ring). GTP hydrolysis then sends a signal to as-yet-unidentified effectors to proceed with fission. So the GTPase domain has to

do a balancing act: too rapid GTP hydrolysis would prevent ring formation or block recruitment of the pinchose machinery to the GTP-bound ring; but a complete failure of GTP hydrolysis would not activate fission.

Presumably, if the neck of the vesicle is too large this will preclude efficient lateral dynamin associations (dynamin rings do not form on templates larger than 30 nm across). One end of the spiral cannot connect up with the other, and the GTPase will not be activated. By sensing the dimensions of neck constriction dynamin acts as a checkpoint, in effect coordinating the last step of budding. Sever *et al.* predict that if dynamin forms an incomplete ring it should have low GTPase activity (to allow recruitment of the fission machinery), and that only after the rings stack should there be a dramatic increase of GTPase activity.

But the new model is not without problems. We must assume that the GTPase mutants studied previously did not just affect the rate of GTP hydrolysis, but that they also inhibited the interaction with unknown downstream effectors. We must also explain why Sever *et al.* find that endocytosis is increased. Perhaps the delay in GTP hydrolysis allows a deeper dynamin spiral to form, increasing the number of binding sites for downstream effectors or prolonging their activation. And we must find out why dynamin changes shape on binding GTP — does this change allow the fission machinery to bind, or does it allow the dynamin spirals to interact, leading to rapid GTP hydrolysis?

Although one possible conclusion is that dynamin is not the true pinchose, the system could, in theory, work with dynamin alone. The pinching would have to happen after the conformational change that occurs on binding GTP, which has been assumed to cause vesicle scission. But there could be a second, more elusive, conformational change after GTP hydrolysis, before the dynamin spiral falls apart. And perhaps the GTPase defect in Sever and colleagues' mutants allows a longer spiral to assemble, which is why the fission reaction is faster. □

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Daedalus

New eyes

The human eye, as the spectacles industry shows, is astonishingly imperfect. We cannot blame evolution; our primitive ancestors must have had perfect vision for their spacious world. But then civilization invented craft-work, reading and other close-up activities. Young eyes had to adapt to very short distances. Daedalus surmises that such repeated stresses on the lens of a growing eye give rise to short-sightedness, and possibly astigmatism too.

Now almost every organ of the body is flexible enough to adapt to the demands made on it. Why does the eye alone persist in useless errors such as astigmatism? One theory blames the invention of spectacles. Once an optometrist has prescribed corrective lenses for your eyes, they are fixed in their imperfection. Any changes they might make will make your vision worse; so they have no 'motive' for improvement. Daedalus now has a remedy.

These days, he points out, children (and indeed many adults) spend most of their waking hours in front of TV, computer or games-machine video screens. So he is devising a screen which appears at optical infinity, like the 'head-up' display in a fighter cockpit. For the dwindling numbers of young book-readers, a similar infinity book-viewer should also be possible. Users of these devices should reach adult life in much better ophthalmic shape.

To correct remaining errors, Daedalus recalls a flexible spectacle lens he once invented, made of the piezoelectric polymer PVDF. It was deformed into the desired shape by a pattern of voltages applied through transparent electrodes and maintained by a small battery-powered circuit. As the wearer's eyes altered, his optometrist simply updated the voltages. Daedalus now reckons that these cunning spectacles should be set so as to under-correct the wearer's vision slightly. His eyes will therefore have an incentive to 'learn' the slight adjustment needed for sharp vision. When they have done so, the 'training glasses' will be under-corrected again, provoking further adaptation. In due course the glasses will be reduced to plane lenses. They can then be discarded, for the wearer will have perfect vision.

Thus the optimization mechanism, which our eyes must possess, will be put to good use at last. We will recover the keen vision of our distant ancestors, at least until middle age when our lenses start to harden. Even then, Daedalus's training glasses may be able to encourage a valuable extra degree of optical flexibility.

David Jones

Obituary

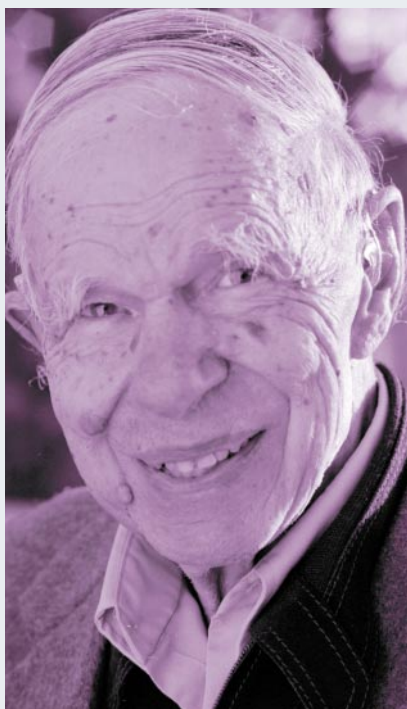
Glenn T. Seaborg (1912–99)

Prophet of the nuclear age, who co-discovered ten new elements including plutonium

Glenn T. Seaborg, who died on 25 February 1999, will be remembered not only for his many accomplishments in science but also for his participation in the associated political structures. He was truly a giant of science, who enjoyed the adventures that were a hallmark of the research of his time. I think it is fair to say that he was the first nuclear chemist, for he went well beyond being a radiochemist. He and his dozens of students applied the tools of radiochemistry to fundamental research in nuclear physics that lay beyond the reach of the physicists of his day. By the time he and his colleagues had discovered plutonium and proved that it was fissionable with slow neutrons, he was no longer simply a chemist. The top-secret Manhattan Project had been started with the aim of producing plutonium for nuclear weapons purposes and his life would be changed forever.

Seaborg was a very intense person who always 'kept his eye on the ball' and seemed to have the knack of doing the right thing at the right time. He was an excellent judge of the abilities of other people, which became crucial when, between 1942 and 1946 he headed a large crew of PhD chemists working on the Manhattan Project at the University of Chicago. Their job was to determine a suitable chemical procedure for isolating the plutonium from uranium that had been highly irradiated by the huge Hanford neutron reactors in Washington state. This was an awesome assignment and he followed the work in every detail, so much so that he suffered nervous exhaustion part of the way through and had to take several weeks off to recover. During the war most of the scientists worked about 60 hours per week (meetings were always held at night), so to relieve the stress Seaborg began playing golf at the nearby Jackson Park golf course. Although his tortured swing was something to behold, it was not unusual for him to get on the green in three strokes and beat his opponents with his short game.

His first love was the expansion of the periodic table, and as soon as he felt that the chemical separation of plutonium was under control he decided that the next step was to look for new elements beyond plutonium. He had two options: one was to use the newly built Hanford reactors to add neutrons to the plutonium until it β-



decayed to a new element with the atomic number 95; the other was to use helium ions from the cyclotron in Berkeley to make a new element with the atomic number 96. He used both methods and eventually succeeded in both endeavours. But there were two big problems. One was that the instruments were relatively primitive, so their sensitivity was limited. The other was that, although the chemistry of plutonium was well-known by this time, the chemistry of the new elements was a complete mystery. These factors meant that little progress was made during many months of effort.

Seaborg finally made a breakthrough when he formulated his actinide theory, which required a major realignment of the periodic table. For many years Seaborg's idea that these new elements were part of a transition series like the lanthanides was opposed by other chemists, but in the end his insight enabled the chemical identification of elements up through lawrencium-103. I was a member of the team that worked on these elements and can testify to the dedication that he applied to this research.

His work in the transuranium field led inevitably to the Nobel prize in 1951 and then to public service outside Berkeley. In 1961 President Kennedy asked him to be chairman of the US Atomic Energy Commission, launching his career into the international arena. The position was a very responsible one, both for science and

for world politics, and he diligently applied himself to it for ten years under three presidents: Kennedy, Johnson and Nixon. One of his most far-reaching actions was his espousal, while chairman and afterwards, of the Comprehensive Test Ban Treaty. Although that job was a very demanding one, he always had time to talk about the science that was dearest to his heart, the transuranium elements, and I found that I always had access to him and his assistants. It was because of him that the 'transplutonium' programme in the US was launched and carried through to the point where the heaviest elements would become available for various kinds of chemical and physical research; it is thanks to this programme that today we have an extensive knowledge of these rare elements. Part of his legacy is the production of large quantities of pure plutonium-244. This was the target material used recently at the Joint Institute for Nuclear Research in Dubna, Russia, during the production of element 114, the first truly superheavy element.

Seaborg had the valuable habit, starting when he was only eight years old, of keeping a journal that he maintained throughout his entire life. The journal enabled him to keep track of people, events and data to complement his fabulous memory. In 1963 I was one of ten scientists who, under his leadership, spent two weeks touring the laboratories of the Soviet Union. I found the trip exhausting, and was amazed that at the end, when Seaborg gathered us all together at the United States embassy in Moscow to discuss the writing of our report, he had the best grasp of the detailed information that would go into it.

When Seaborg returned to Berkeley, after a long stint in the 'jungle' of Washington D.C., it would not have been surprising if he had settled down to a less hectic existence, but that was not to be. He immediately applied himself to converting what he had learned in those early years into his published journals, which are a marvel of detail. He also became an advocate for nuclear power, a very unpopular position to take. He felt very strongly that the most significant achievement of his tenure at the Atomic Energy Commission was the growth of the civilian nuclear-power programme, and he argued that such a programme would be best for the world in the long run. He could still be right.

Albert Ghiorso

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Exposing the human *nude* phenotype

The recent discovery of the human counterpart of the *hairless* mouse phenotype¹ has helped our understanding of the molecular genetics of hair growth. But there are no reports of a defect in the human homologue of the best known of the 'bald' mouse phenotypes, the *nude* mouse². This may be because affected individuals are so gravely ill from the accompanying immunodeficiency that their baldness goes unnoticed. We have carried out a genetic analysis that reveals a human homologue of the *nude* mouse.

The *nude* mouse is characterized by a congenital absence of hair and a severe immunodeficiency², resulting from mutations in the *whn* (*winged-helix–nude*; *Hfh11tm*) gene, which encodes a member of the forkhead/winged-helix transcription factor family with restricted expression in thymus and skin³. The simultaneous occurrence of severe functional T-cell immunodeficiency, congenital alopecia and nail dystrophy (MIM database no. 601705) in two affected sisters led to the recognition that the clinical phenotype was reminiscent of the *nude* mouse⁴. We therefore investigated whether this syndrome represents the human counterpart of the *nude* mouse phenotype.

We obtained DNA samples from members of the sisters' family in a small village in southern Italy. The affected sisters were born with a complete absence of scalp hair (Fig. 1a), eyebrows and eyelashes and had dystrophic nails, and no thymic shadow was evident upon X-ray examination. The first affected child revealed a striking impairment of T-cell function shortly after birth, and died at the age of 12 months. Her sister had similar immunological abnormalities, but bone-marrow transplantation at five months of age led to full immunological reconstitution, although the alopecia and nail dystrophy are still present⁴.

We performed linkage analysis using microsatellite markers near the human *WHN* locus on chromosome 17, and found a lod score of 1.32, suggestive of linkage. We then sequenced the human *WHN* gene⁵ and found a homozygous C-to-T transition at nucleotide position 792 of the *WHN* cDNA (GenBank accession no. Y11739) (Fig. 1b). This leads to a nonsense mutation at residue 255 (R255X) in exon 5, and predicts the complete absence of functional protein as a result of nonsense-mediated decay of messenger RNA.

Because the proband's bone-marrow transplant was from her brother, we examined her leukocyte DNA both before and after the graft for the presence of chimaerism. Genotyping the proband before

the transplant showed that her leukocyte DNA was homozygous only for the mutant allele (Fig. 1c). Four years after the transplant, we detected the haplotype specific for the wild-type paternal *WHN* allele received from the brother, as well as the mutant allele, indicative of chimaerism. Gender determination revealed that the proband's leukocyte DNA was genotypically XX before the transplant, and the brother's DNA was XY. Afterwards, the proband's leukocyte DNA was found to be XY (Fig.

1c), providing evidence of long-term engraftment and expansion of the bone-marrow graft.

The *WHN* gene encodes a transcription factor, which is developmentally regulated and directs cell-fate decisions⁶. In mammals, *whn* is expressed specifically in the epithelial cells of the skin and thymus, where it helps to maintain the balance between growth and differentiation^{7,8}. Recent evidence⁹ has highlighted the importance of the thymic microenviron-

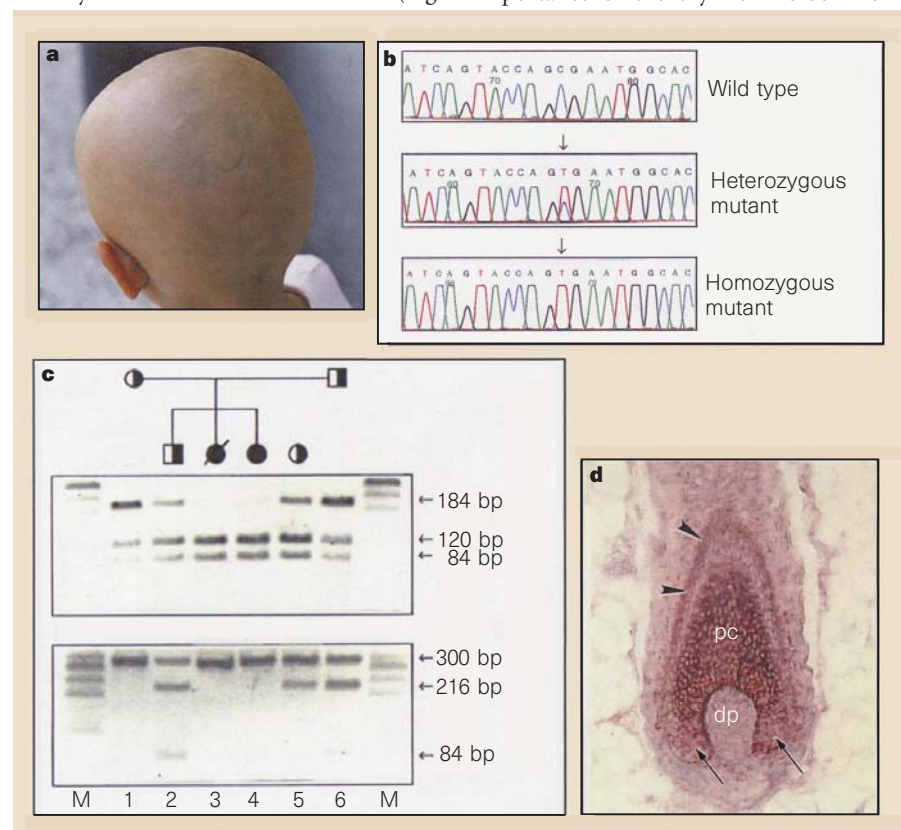


Figure 1 Molecular analysis of the human *nude* phenotype. **a**, A five-year-old child with congenital alopecia and T-cell immunodeficiency. **b**, Sequence analysis of a nonsense mutation in exon 5 of the *WHN* gene. Top, homozygous wild-type sequence from an unrelated, unaffected control individual. Middle, sequence from a heterozygous carrier of the mutation R255X; arrow indicates a double T+C peak. Bottom, homozygous mutant R255X sequence from the affected individual; arrow indicates mutant T only, leading to a C-to-T transition (OGA to TGA) and a substitution of an arginine residue by a nonsense mutation. **c**, Restriction-enzyme digestion confirms the mutation. The mutation introduced a restriction site for *Bst*I and, after digestion of the 184-base-pair (bp) polymerase chain reaction (PCR) product containing exon 5, the product generated from the mutant allele should cleave into two bands of 120 and 64 bp. Top, the unaffected parents and brother had three bands of 184, 120 and 64 bp (lanes 1, 2 and 6), indicating that they were heterozygous carriers of the mutation R255X. Both patients had only the two digested bands of 120 and 64 bp (lanes 3 and 4), consistent with the presence of the mutation in the homozygous state. Bottom, evidence for long-term engraftment of the bone-marrow transplant. Gender determination of family members revealed a genotypically XX pattern of an undigested 300-bp band in the mother (lane 1) and affected patients (lanes 3 and 4), and a genotypically XY pattern consisting of the 300-bp band and two additional bands of 216 and 84 bp, indicative of the Y chromosome, in the brother (lane 2) and father (lane 6). Lane 5, peripheral blood leukocytes from the patient after the transplant, demonstrating an XY genotype and the presence of the normal *WHN* allele, providing evidence for fraternal chimaerism and persistence of the graft. M, size markers. **d**, *WHN* mRNA expression in normal human scalp skin. In the hair bulb, *WHN* mRNA is localized to the differentiating cells of the hair follicle precortex (pc) and the innermost cell layer of the outer root sheath (arrowheads); the dermal papilla (dp) fibroblasts and hair matrix below the level of Auber (small arrows) remain negative for *WHN* mRNA.

ment in determining the T-cell repertoire, as both positive and negative selection of developing T cells depends on cell-cell interactions with the thymic epithelium. In *whn*-knockout mice, the defect has been localized to the differentiating thymic microenvironment rather than to a defect in the developing T cells⁷. The proband was free of infections for four years after the bone-marrow transplant, indicating that T-cell function was at least partly restored. This is probably due to mature T cells of donor origin, although we cannot exclude the possibility that positive selection of T lymphocytes occurs in the periphery despite the mutated *whn* gene.

Our findings provide evidence of a human immunodeficiency caused by a gene expressed not in haematopoietic cells¹⁰, but in specific epithelial cells. In the human hair follicle, expression of *WHN* is sharply demarcated in defined cell populations (Fig. 1d). Although *nude* mice appear to be completely naked, the dermis contains a normal number of hair follicles, but they are incompletely developed. The fact that only short, bent hairs occasionally emerge from the epidermis is thought to result from impaired keratinization¹¹. Together with the *hairless* gene¹, our finding extends the evidence implicating cell-type-specific transcription factors in hair-follicle cycling and morphogenesis, and indicates that baldness is an extremely complex phenotype.

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Putting nuclear-test monitoring to the test

On the 22 August 1998, 0.1 kilotonnes of conventional explosives was fired underground at the nuclear test site of the former Soviet Union at Degelen Mountain in eastern Kazakhstan. This explosion, which we refer to as K980822, was carried out by the Republic of Kazakhstan, in cooperation with the United States, to seal tunnels at the site. It provided an opportunity to assess the network of seismological stations being set up as part of the International Monitoring System (IMS) to verify the Comprehensive Test Ban by detecting and identifying low-magnitude disturbances. Despite its low yield, short-period signals from the explosion were recorded up to 88° away. The seismograms recorded are sufficient to identify the disturbance as suspicious and, if the test-ban treaty were in force, could have led to a demand for an on-site inspection to determine whether a nuclear test had taken place.

The prototype International Data Center at Arlington, Virginia, detected short-period P-wave signals from the explosion at ten stations (Fig. 1), and estimates of the epicentre are within 12 km of the true epicentre. (The International Data Centre proper, which will provide data from the IMS to allow signatories to verify the test ban, is being set up in Vienna.) A weak P signal was also detected at Alice Springs, Australia (Fig. 1). The body-wave magnitude (m_b) was estimated to be 3.8. There were no observations of long-period (about 20 s) surface waves from the explosion.

The absence of surface waves at Zalesovo in the Russian Federation, only 6° from the epicentre, does not suggest an earthquake. For an earthquake of m_b 3.8, the surface-wave magnitude (M_s) is on average around 3.5, whereas for underground explosions at Degelen Mountain, $m_b - M_s$ is usually greater than one magnitude unit. Failure to detect a surface wave at Zalesova implies that $M_s < 2.3$, so the disturbance seems explosion-like. But some earthquakes in eastern Kazakhstan are 'anomalous events' in that, on the $m_b - M_s$ criterion, they look like explosions^{1,2}, so this criterion alone is insufficient. These earthquakes are usually easily identified because clear reflections (echoes) from the free surface (pP and sP), which travel from source to reflection point as P and S waves, respectively, and then on to the recording station as P waves, are seen, implying source depths of around 20 km, deeper than the maximum at which an explosion could be buried^{1,2}.

Of the seven stations that detected P-waves at long range (distances of more than 30°), most show simple signals that are typ-



Figure 1 Azimuthal equal-area projection of the Earth, centred on the epicentre of K980822, showing the IMS stations that recorded seismic waves. Origin time, 05:00:18.94 UTC; estimated origin time, 05:00:18.7 UTC. Epicentre, 49.7667° N, 77.9908° E; estimated epicentre, 49.7566° N, 77.8273° E. Depth, 0.140 km. Firing medium, granite. Explosive, 100 tonnes of Granutoll. Estimates are from the prototype International Data Centre's *Reviewed Event Bulletin*. ARCES, Karasjok, Norway; ARU, Arti, Russian Federation; ASAR, Alice Springs, Australia; BGCA, Bangui, Central African Republic; EKA, Eskdalemuir, UK; HFS, Hagfors, Sweden; ILAR, Eielson, Alaska, USA; NOA, Hamar, Norway; NRIS, Norilsk, Russian Federation; PDY, Russian Federation; ZAL, Zalesovo, Russian Federation.

ical of explosions, with the amplitude in the first 2–3 s after onset being significantly larger than the coda. The seismograms from three seismometer arrays detecting the disturbance are shown in Fig. 2. To confirm the presence of the signals, each array is considered as two sub-arrays with roughly equal numbers of elements, and the summed seismograms from each sub-array (the semi-sums) are formed separately. The semi-sums filtered in a 1.5–3.0 Hz pass-band are then multiplied together point by point. The product for the three stations is positive, confirming the presence of coherent arrivals (Fig. 2).

If the seismograms had been from an earthquake deeper than a few kilometres, then surface reflections would be expected at some of the stations. But the radiation pattern of the double-couple source — the accepted model of an earthquake — is not uniform, so the absence of visible surface reflections may be due to the orientation of the double-couple resulting in weak surface reflections relative to *P*. This can be tested by searching for orientations of a double-couple source that are compatible with the relative amplitudes of *P*, *pP* and *sP* and their polarities, where these can be observed unambiguously^{4,5}. In practice, the method involves placing bounds on the amplitudes of *P* and possible surface reflections to allow for uncertainties in the measurements (Fig. 2). No orientation of a double-couple is found that is consistent with these bounds.

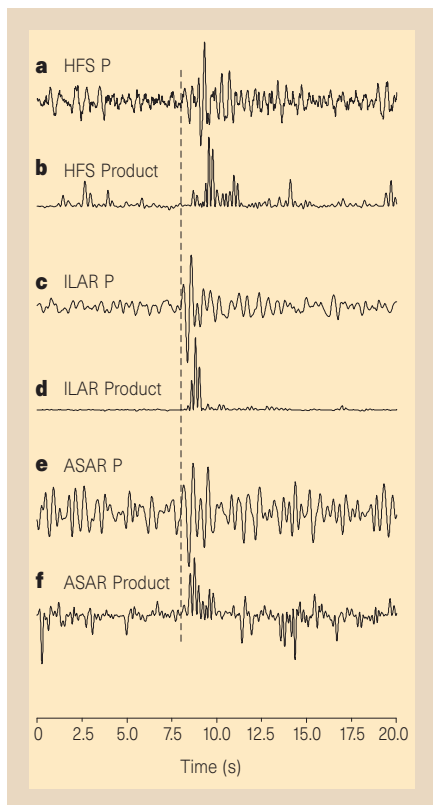


Figure 2 Short-period P-wave seismograms from K980822. Recording stations: **a, b**, HFS, $D=36.8^\circ$, $A=311.3^\circ$; **c, d**, ILAR, $D=60.7^\circ$, $A=20.2^\circ$; and **e, f**, ASAR, $D=88.4^\circ$, $A=130.4^\circ$. D is the epicentral distance and j is the source to station azimuth. **a, c, e**, Seismograms have been processed using an optimum filter³ to attenuate low-frequency noise. An additional lowpass filter cutting off at 3 Hz has been applied to the ASAR seismogram. **b, d, f**, Product of the semi-sums. Amplitude ranges were used to test for compatible double-couple sources. HFS, P, 1.5–3.0; pP, 0.0–2.0; sP, 0.0–2.0. ILAR, P, 3.0–4.0; pP, 0.0–1.0; sP, 0.0–1.0. ASAR, P, 1.0–2.5; pP, 0.0–1.5; sP, 0.0–1.5. All amplitudes are in arbitrary units. Polarities are assumed to be unknown except for P at ILAR, which is taken to be positive.

This indicates that the source is so shallow that surface reflections arrive within 1.7 s of the onset, which should arouse suspicion.

The analysis shows that explosions with yields well below 1 kilotonne can be recognized as suspicious. In contrast, the Non-Proliferation Experiment⁶, in which 1 kilotonne of conventional explosive fired at the Nevada Test Site in the United States on 22 September 1993, was less well recorded at long range. Its m_b was 4.1, compared with the 4.6 predicted for an explosion at Degelein Mountain with the same yield.

These results possibly overstate the ability of seismologists to identify small explosions. Knowing when and where the explosion took place means that the signals can be interpreted with more confidence than would be possible when analysing signs of a possible disturbance for which the only information is the seismograms. But

when the IMS and the International Data Centre are fully operational, such disturbances will be recorded by more stations, with wider azimuthal coverage, increasing the confidence of the interpretation.

Anyone attempting a clandestine test could be sure of getting away with it only by using yields well below that used here or by attempting to muffle, or decouple, the test by firing the explosion in a large cavity. A decoupled test using conventional explosives is planned for the site next year. We await the results with interest.

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Seeing movement in the dark

Our visual world is greatly reduced at night. Spatial and temporal resolution are poor, contrast sensitivity is diminished, and colour vision is totally absent¹, as rod photoreceptors are used rather than the cone photoreceptors that operate during the day. Many aspects of rod vision, including spectral, contrast and flicker sensitivity, have been studied in detail¹, but motion perception has been largely ignored². We find that motion perception using rods is impaired, with moving objects appearing to be slower than they are during cone vision.

Rod monochromats, who lack cone vision and are totally colour-blind^{3,4}, often report problems when dealing with moving objects. To find out whether such problems are encountered by normal observers when restricted to rod vision, we compared the speed of moving objects as seen through rods and through cones.

We used a standard cathode-ray-tube monitor and conditions chosen to isolate either rods or cones. These monitors have only three independent parameters, one each for the red, green and blue phosphors, whereas the human visual system has four: one for the rods and one each for the red, green and blue cones. A direct comparison is therefore impossible in observers with normal colour vision because we cannot stimu-

late the rods in isolation while silencing all three cone classes. We overcame this difficulty by using observers with red–green colour-blindness (dichromats, about 2% of the male population). Our observers were deuteranopes, who lack functional green cones⁵. For such observers, who have normal motion perception for luminance-defined stimuli⁶, it is possible selectively to excite rods while silencing activity in red and blue cones, and to excite red cones while silencing the rods and blue cones.

We used standard psychophysical methods to determine the physical speed at which stimuli selectively activating rod and cone photoreceptors appear to move equally fast. Rod-detected motion stimuli appear to move at about 75% of the speed of a reference motion stimulus detected only by red cones, and red-cone-detected motion stimuli appear to move at about 130% of the speed of a rod-detected reference motion stimulus (Fig. 1). This is also the case at several dimmer light levels, for which the relative visibilities of the rod and cone stimuli vary considerably. There is a subtle but significant dependence on contrast^{7,8}, with stimuli of higher contrast appearing to move faster, regardless of whether they are detected by rods or cones.

What causes the poorer performance of the night-vision rod system? A cortical explanation, in which rod- and cone-detected motion are analysed by different systems, is unlikely because the overall dependence on contrast was identical for rods and cones⁸. More compelling is a retinal explanation, in which the slowing may be caused by the spatial and temporal averaging in the distal rod visual system^{1,9}. Such low-pass filtering of the visual input signal

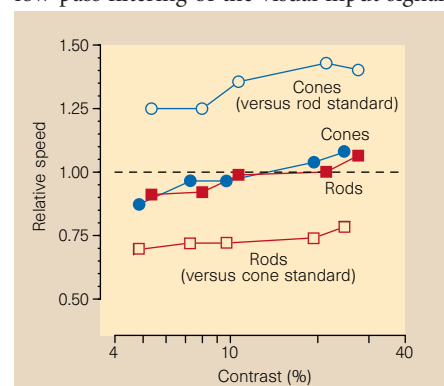


Figure 1 Relative movement of rod and cone stimuli. Perceived speed is shown as a function of percentage rod or red-cone contrast. Reference and comparison stimuli activated either the same (filled symbols) or a different (open symbols) class of photoreceptor (rods or red cones). Space-time-averaged luminance was $0.92 \log \text{ cd m}^{-2}$, a level at which both rods and cones are active. On each trial, observers judged which of two moving sine-wave gratings (1 c deg^{-1}) moved faster. Gratings were presented simultaneously for 1 s and moved in opposite directions. For more details, see ref. 8.

will affect the output of motion detectors driven by rod signals. As a result, the relative signal at slow velocities will be higher than at fast velocities for rod vision, so the stimuli will appear slower.

The use of artificial lighting can help to avoid the disadvantages of rod-mediated vision. Indeed, most tasks can now be performed as well by night as they can by day. But driving at night may be an important exception. The region illuminated by the car headlights is processed mainly by the cones, whereas on a poorly lit road the remaining visual field is in the dark and is processed mainly by rods. The region in the dark includes the sides of the visual fields, where transitory motion signals are largest. This might lead to an underestimation of the speed of movement, which in turn might elicit a compensatory — and possibly fatal — increase in speed¹⁰.

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Sexual propagation by sponge fragments

Habitat fragmentation means that many species occur in discrete populations¹, so it is important for sessile species to colonize new areas. It has not been clear how sponges whose larvae disperse over short distances achieve this. Fragments may break off sponges as a result of physical and biological disturbance and are then dispersed by currents and recruited as independent individuals or colonies^{2,3}. Local populations are expected to have high genetic relatedness as a result, but most sponge populations have high levels of genetic variability^{4,5}. We suggest that this discrepancy results from an interaction between fragmentation and sexual reproduction.

We studied reproduction in *Scopalina lophyropoda*, a sublittoral demosponge with

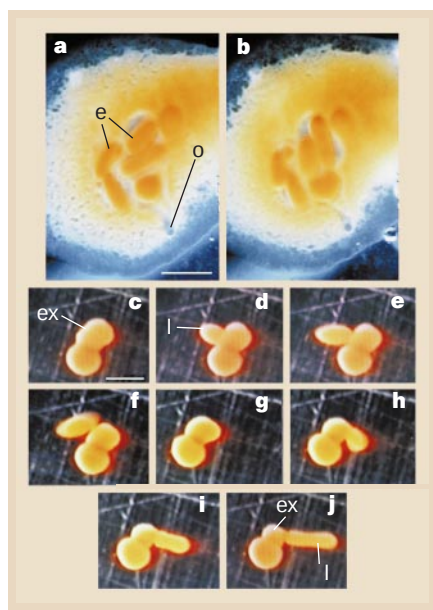


Figure 1 Time-lapse observations of embryogenesis and larval release. **a, b**, A small sponge reorganized from an explant. Six embryos (e) are incubated in a chamber connected to the exterior through an oscular tube (o). Note the differences in size and repositioning of embryos in the 90 min between pictures. **c–j**, Time-lapse observations over 93 min of two embryos escaping as free-swimming larvae from an unattached explant (ex), which is in contact with another non-histocompatible fragment. Scale bars, 1 mm.

populations sparsely scattered over the western Mediterranean⁶. In this species, embryos are incubated and lecithotrophic parenchymella larvae are released from mid-July to late August. Because the planktonic phase lasts only 2.5 days, most larvae are believed to recruit locally. However, the dispersal potential of these larvae is significantly increased by the fragmentation of brooding adults as a result of wave action and foraging by fish and echinoderms.

Tissue shredded artificially reorganizes into dense balls, in which internal epithelia and choanocyte chambers dissociate and most specialized cells regress to an amoeboid, archaeocyte-like morphology. If detached fragments lie immobile on the substratum for a few days, they reattach and reorganize as functional sponges. When fragmentation occurs in reproductive sponges, fragments as small as 1 to 2 mm across may contain several developing embryos. Because development requires nourishment from specialized nurse cells through a placental-like membrane⁷, we expected that the embryos would be reabsorbed and their energy reallocated to reorganize the colony. Instead, embryos were nourished by cells in the tissue fragment, where they completed development to free-swimming larva.

We used forceps to disintegrate sponges in early and late embryogenesis (one month before and 10 days after the onset of larval

release), and randomly selected explants (balls of tissue) 1–2 mm across ($n = 80$ per group). We found that 52.5% of the explants obtained in early embryogenesis remained embryogenically active and released a total of 102 free-swimming larvae (between 0 and 7 per explant) over a 28-day period. The first two larvae were released on days 4 and 8, before the explants reorganized as sponges. The remaining embryos were incubated for 14–28 days in a translucent, atrium-like chamber formed once explants had reorganized into sponges (Fig. 1a). Nearly mature larvae moved around inside the chamber for several days before being released through an oscular tube (Fig. 1a,b). The atrium then lost its structure and the oscules regressed to being perforations.

When mother sponges in late embryogenesis were shredded to obtain explants, most mature embryos escaped as free-swimming larvae. Only 6.25% of explants contained developing embryos, and just seven larvae were released over a 28-day period, escaping from unattached explants in all cases by crawling through the disorganized mass of cells in the explant (Fig. 1c–j).

Explant attachment took between 3 and 24 days in calm water, with just 2.5% remaining unattached after 28 days, and these were apparently still viable. There were no significant differences in time to attachment between explants that carried embryos and those that did not. It is difficult to estimate possible dispersal distances before attachment, but large sponge fragments can disperse rapidly over several kilometres during heavy storms³.

Our results show that even small fragments often carry the essential functional elements for reorganizing and nourishing embryos. Our findings also indicate that fragmentation may interact with sexual reproduction. This means that the dispersal capacity of sexually produced propagules is maximized by the additional dispersal of the asexual propagule. The dispersal of embryo-bearing fragments also maximizes the chance that several distinct genotypes will reach a new area, increasing the chance of establishing new populations.

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Bantu banter and African roots

An African Classical Age: Eastern and Southern Africa in World History, 1000 BC to AD 400

by Christopher Ehret
University of Virginia Press/James Currey:
1998. 341 pp. \$45, £35

Jared Diamond

Between 1000 BC and AD 400, the prehistory of sub-equatorial Africa was transformed by the expansion, over most of the region, of farmers speaking Bantu languages. A new book by Christopher Ehret transforms our understanding of this event. Ehret gives us rich insights into big questions of world history, while also showing how prehistory can be deciphered with a method termed linguistic archaeology.

The central problem of sub-equatorial African history is as follows. Today, native peoples of the region speak languages belonging to six groups: Bantu (by far the most widespread), Central Sudanic, East Sahelian, South Nilotic, South Cushitic and Khoisan. Different traditional peoples of the region practise different mixes of farming, herding and hunting–gathering. Farmers use different mixes of West African and Indonesian wet-climate crops and Sahel and Red Sea dry-climate crops. None of the important crops or domestic animals are native to sub-equatorial Africa; all must have been brought in from outside. What peoples invaded, at what times and from what directions, and which crops, livestock and other cultural baggage did each invader bring? What advantages enabled the Bantu to emerge from that human melting pot with a much wider distribution than the other five groups?

If only we had tape-recordings of conversations from African villages 3,000 years ago! Analysis of those recordings would go a long way towards answering our questions. In the absence of recordings, one can still try to reconstruct some by using linguistic archaeology. One thereby aims to deduce cultural attributes, population interactions and migrations in the preliterate past, not by the usual archaeological methods of excavation, but by analysis of modern languages. Among the members of a modern language family, the analysis maps the distributions of word roots that were evidently borrowed from various other languages at various past times.

Consider these examples from Indo-European languages that will be more familiar to most *Nature* readers than Bantu languages. The word for ‘sheep’ is similar in most Indo-European groups: ewe, *ovis*, *avis*, *ois* and *oi* in English, Latin, Sanskrit, Greek and Old Irish, respectively. The same is true for ‘fart’: fart, *pardate*, *perdet*, *perdzu* and



Speech marks: linguistic archaeology can track how this Khoisan woman's language...

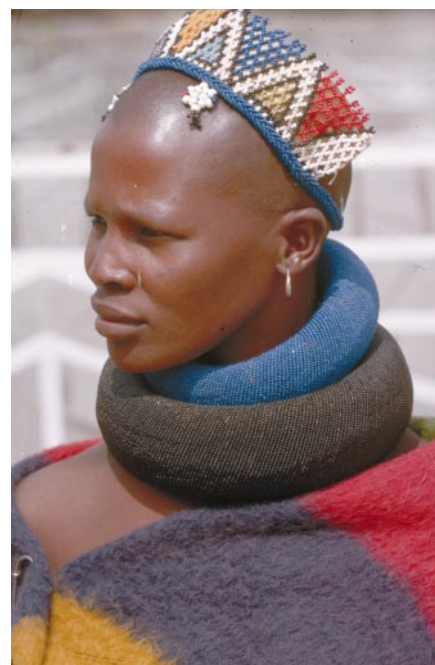
perdo, in English, Sanskrit, Russian, Lithuanian and Greek, respectively. These shared words permit reconstruction of the roots ‘owis’ and ‘perd’ for sheep and fart in the ancestral proto-Indo-European language spoken at some time in the preliterate past. But the word for ‘gun’ is entirely different in different Indo-European languages: gun in English, *fusil* in French, *Gewehr* in German, *ruzhyo* in Russian, and so on. The inference is obvious: proto-Indo-Europeans, who probably expanded out of West Asia around or before 4000 BC, already had sheep and farts, but no guns, so that different modern Indo-European languages share ancient inherited roots for sheep and fart but independently coined new words for guns upon their recent invention.

In Ehret's hands, linguistic archaeology yields even clearer and richer conclusions when applied to sub-equatorial Africans than to Indo-Europeans. The reasons for this are: sub-equatorial African languages are so diverse and distinct (six stocks and many substocks); the main population expansion was more recent in sub-equatorial Africa (1000 BC–AD 400) than in West Eurasia (7000–4000 BC); and, as a result, ancient sub-equatorial language stocks are still mostly well represented by living languages, whereas many ancient European stocks are now extinct and can only be inferred from strange word roots in existing languages (for example, Greek words evidently borrowed from a now-extinct pre-Indo-European language of Greece). In broader or narrower subsets of

the now-dominant Bantu language family, Ehret recognizes words evidently borrowed from each of the five other stocks.

For instance, all Bantu substocks share similar words for wet-forest animal species such as leopard and hippopotamus, but different Bantu substocks have different words for East African dry-grassland species such as zebra and ostrich. This suggests that the Bantu who colonized sub-equatorial Africa arose from a wet-forest environment and did not encounter East African dry grassland until later in their history. While this principle is familiar to linguists, I am impressed by the wealth of detail that Ehret extracts when he applies it to African languages. For example, he demonstrates that, in their eastwards expansion, the Bantu interacted successively with Central Sudanics, East Sahelians, South Cushites, South Nilotes, Khoisan and Indonesians in approximately that sequence (because Bantu words for items such as sorghum, iron, cow, black and white cow, click consonants and chickens are loan words from these language stocks, respectively, but are confined to successively narrower subsets of Bantu languages).

As one example of Africa's lessons for world history, the spread of food production around the world from its few prehistoric centres of origin is thought usually to have involved the spread of farmers and herders themselves, bringing their crops and livestock with them and replacing local hunter–gatherers. The best-attested example known to me of the alternative explanation — local hunter–gatherers



... was upstaged by the language spoken by this Bantu woman.

CORBIS/PETER JOHNSON

CORBIS/CHARLES & JOSETTE LINARS

acquiring livestock or crops from food-producing neighbours — comes from Southern Africa, where Khoisan hunter-gatherers acquired sheep and cattle to become the ancestors of modern so-called Hottentot herders. Ehret identifies loan words for 'ram', 'young ram', 'milk ewe', 'grain food' and 'porridge' in a reconstructed early Khoisan language. Astonishingly, though, these loan words have not been borrowed from Bantu languages, which are now the sole native farming languages in Africa south of the Zambezi River, but from East Sahelian languages, now confined to regions over 1,000 km north of the Zambezi. This suggests a vanished former thrust of East Sahelians far south of their modern distribution. Here is a hypothesis awaiting testing by archaeologists: will excavations yield any evidence of East Sahelians 2,000 years ago living as far south as Malawi, Zimbabwe and Botswana?

Ancient East Africa was evidently a melting pot of peoples adapted to different lifestyles: the Bantu with their West African wet-forest root crops and goats; Central Sudanics and East Sahelians near the West Rift Valley, with their dry-country grain crops, sheep and cattle; South Cushites in the Eastern grasslands and South Nilotes at high altitude, with their cattle; and Khoisan hunter-gatherers in areas unsuitable for agriculture.

As each group of food producers trickled into East Africa, they gravitated towards environments similar to those they had left — just as, during the European colonial era, Spanish immigrants preferred warmer areas of the New World, while British immigrants preferred cooler areas of North America, Australia, South Africa and the Kenya highlands. Bantu latecomers were able to gain a foothold in East Africa because their wet-forest agriculture let them farm areas unsuitable for the dry-country grain agriculture and herding of the other food-producing groups.

Once the Bantu had added grain crops, cattle, sheep and iron tools to their original inventory, they found themselves in possession of an economic package that was unstoppable in the subequatorial Africa of those times. Within a few centuries of reaching the east coast of Tanzania, Bantu food-producers advanced southwards for several thousand kilometres, through areas only thinly populated by Khoisan hunter-gatherers, to reach South Africa.

Why, out of this human melting pot, was it the Bantu and not one of the other food-producing groups that developed the unstoppable package? Ehret hints at possible cultural factors — the wild card of environmental historians. Before the Bantu arrived, Khoisan hunter-gatherers had coexisted for a long time in East Africa with Sudanic, Sahelian, Nilotic and Cushitic farmers, because most of those food-producers did not hunt. The Bantu did hunt, so there was no room left for separate Khoisan hunter-gatherer

societies, which were speedily out-competed and eliminated. But why did not the Sudanics and those other peoples hunt and eliminate the Khoisan earlier? And why was it the Bantu who added dry-country crops to their wet-country inventory and emerged the victors, instead of all those dry-country food-producers adding Bantu wet-country crops to their own repertoire and emerging the victors? Why, in East Africa's environmental mosaic, was there such an extensive melting-together of economically distinct societies, whereas India's superficially similar environmental mosaic continued to harbour specialized groups of farmers, herders and hunter-gatherers until modern times?

Now that Ehret's linguistic analyses have brought these questions into focus, archaeologists can test his hypotheses for Africa itself, comparative historians can use African history to illuminate world history, and comparative linguists can imitate his approach elsewhere in the world. □

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For Charlie and Nick

The Shape of Actions: What Humans and Machines Can Do

by Harry Collins and Martin Kusch
MIT Press: 1998. 212 pp. \$26.50, £15.95

Bruce Mazlish

As the human species enters a new millennium, it is engaged in a fateful intensification of a long-standing question: is there any difference between humans and machines, and, if there is, what is it? A few thousand years ago, Chinese automata posed the issue physically, with mechanical figures putatively looking and functioning like their makers. How was one to tell the difference? The Greek construction of automata also gave actual features to what we can call the 'android look'. By the seventeenth century, René Descartes had posed the question philosophically in his classic *Discourse on Method*, claiming that there were two certain ways to recognize the difference. Put in the simplest terms, his two criteria for distinguishing between man and machine were that the latter has no feedback mechanism ("it could never modify its phrases") and no generalizing reason ("reason is a universal instrument which can be used in all sorts of situations").

Needless to say, the debate raged fiercely in Descartes' time, and has continued ever since. The issue is omnipresent even in such an unlikely place as the childish Oz stories of the early twentieth-century writer Frank Baum. One need only think of the Tin Man, but the mechanical creatures of *Ozma of Oz*

serve as especially fascinating examples. Here we meet the Wheeler, formed like a man but moving on wheels rather than feet, and Tiktok, the machine man who "thinks, speaks, acts" and "does everything but live". Having been fitted with a brain, he claims the power of ratiocination. What then distinguishes him (it?) from humans? We are, clearly, only a short step away from devices with artificial intelligence (AI), where, in practice and not just fictionally, mental activity is added to physical activity as part of the repertoire of mechanical 'beings'.

It is in a context such as the above that we need to place *The Shape of Actions*. One of the authors, Harry Collins, is a sociologist who teaches at the University of Wales. The other, Martin Kusch, is a philosopher who lectures at Cambridge University. Both have many books to their credit, and Collins is known for his work on the sociology of science, and especially for his embrace of the notion of the social construction of science (that is, that scientists do not so much discover what is in nature, as impose certain conventional ideas on it), and the role of tacit knowledge (knowledge acquired through a collective socialization process and irreducible to precise formulae) in the actual doing of science. Together, they claim to be doing "philosophical sociology ... following the tradition of the later [Ludwig] Wittgenstein (1953) and of [Peter] Winch (1958)".

Well, we may ask, what does philosophical sociology have to say about the human-machine relation? The authors' great insight, announced with much fanfare and claim to originality, is a version of the philosophy of action. Their theory of morphology, they tell us, goes like this: "Humans can do two kinds of actions and — like machines — they can merely behave. When humans do *polymorphic actions*, they draw on their understanding of their society; when they do *mimeomorphic actions*, they intentionally act like machines — entities that do not need to understand society ...". Machines, a term they define broadly, the authors conclude, are incapable of polymorphic action. Basically, the rest of their book is a variation on this theme.

Specifically, they apply their analysis to all manner of things — letter writing, golf swings, bike balancing and riding, restaurants (especially McDonald's), armies, and other such examples of 'mechanical' possibilities. Their text is filled with tables and branching trees, which we are encouraged to scramble up and down in seeking to establish the differences between humans and machines. (The boundaries, in fact, as the authors point out, are permeable and shifting.) Everywhere, we encounter types, and types within types: we are told that, typically, there are four types of action, three of which are polymorphic. These three are then divided into "open", "occasional"

and “playful” actions. And so on.

A reader will either like such thinking or not. Either one thinks this ratiocination sheds new light on the human/machine question or one does not. Indeed, the authors themselves admit that readers might wish initially to skip the “densely argued” early chapters. For, in fact, most of the book is implicitly aimed at other philosophers of action — for example, G. E. M. Anscombe, R. M. Chisholm, A. C. Danto and D. Davidson — and is engaged in an internecine tussle. When it does get to apply its philosophy to the machine question, we suddenly realize that it is also aimed at other sociologists of science, such as Bruno Latour. In short, the book may appeal to various in-groups, but may well leave a general reader bored or baffled, or both.

All in all, the book seems like a stitched-together piece of work, rather than an exemplar of interdisciplinarity. Whimsically, it is dedicated to Charlie Chaplin, though no explanation is given for this choice (are we to remember Chaplin in the film *Modern Times* clinging to the hands of a huge clock, trying to stop it; or is it the IBM advertisement that we are to recall?), and to Nick Faldo. For those not in the know, Faldo is a golfer whose Nick Faldo-type swing, we are told later in the book, can be analysed according to the philosophy of morphicity. In the end, the authors’ attempts at humour come dangerously close to falderal (they themselves use puns occasionally).

There are more serious problems. The authors are arguing against an AI position that has already sent up the white flag. Or, to put it another way, they are boxing with a shadow, a ‘straw machine’. Most of the people I know who work with computers and robots no longer, if they ever did, take the strong AI position that humans are simply interchangeable with machines, or vice versa. They are simply getting on with their work, trying to build machine intelligence from the bottom up, so to speak. Work done along the lines of neural nets may be outflanking Collins and Kusch’s position; as they themselves admit, “the most advanced neural nets are at the level of the mollusk”. Well, one might say, such neural nets are not bad for a ‘creature’ that has been ‘evolving’ for only about 50 or so years (or a little more than 150 if we go back to Charles Babbage’s automatic digital computer).

Collins and Kusch are obviously bright men; some of their friends might even say brilliant. But they are barking up the wrong tree. The irony is that they themselves are arguing like computers, breaking each action into separate bits and those into further bits. They have lost sight of the forest for the trees. Of course there are limits to the ability of machines to mimic us (less so to our ability to mimic them, as Collins and Kusch point out). There is nothing, however, in principle,

to prevent computer robots from reproducing themselves with variations, having learning experiences as they move about, and, in short, transforming themselves. Whether one wants to call this ‘evolution’ is a separate matter.

It is in some such development that the future relations of humans and machines will unfold. Not in terms of a philosophy of action that restricts or simply ignores such developmental activity. *The Shape of Actions* is, in the final analysis, a sterile work — general readers will hardly find inspiration in it, and computer workers will simply put it aside. The next millennium will probably be the scene of a continuing great and exciting adventure as humans seek to further explore their differences from machines. I doubt that *The Shape of Actions* will serve as a guidebook. □

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Reasons to be miserable

The Nature of Grief: The Evolution and Psychology of Reactions to Loss

by John Archer

Routledge: 1999. 296 pp. \$31.99, £18.99

Hugh Freeman

My maternal grandfather died two years after the entirely unexpected loss of his wife which, I was later told, he “never got over”. He was still relatively young, but seemed to have lost the will to live. *Post hoc*, but not necessarily *propter hoc*, would probably be John Archer’s view, judging from this comprehensive synthesis of contemporary knowledge of grief.

Britain has made two major contributions to the scientific study of grief: John

Bowlby’s on attachment (which is disrupted by bereavement) and the clinical-community research of Colin Murray Parkes, which revealed what happens in real life. Archer, who is Professor of Psychology at the University of Central Lancashire, has sought to link these fundamental studies with “the modern Darwinian thinking that underpins evolutionary psychology”.

His essential argument, in contrast to the psychiatric approach, is that grief is neither an illness nor a disorder, but a natural reaction to losses of various kinds. It has evolved from simpler versions in animals to include higher-level mental processes which attribute meaning to what has been experienced. He believes that our ideas on the subject have long been in a state of confusion, not least through the uncritical absorption of Freud’s speculations.

This confusion, in Archer’s view, is also the result of approaching the subject from the wrong end — that of specific issues, rather than broad theoretical principles. As to what these principles should be, his answer is essentially those of evolution: for instance, the index of relatedness to the lost person, or their reproductive value — the loss of older children generally causing more intense grief than that of younger ones. Whilst attachment theory does predict that the loss of the closest kin evokes the strongest grief, evolutionary theory provides a reason for the existence of this gradation in the strength of attachment.

Yet what is the initial selectivity that starts off the interaction leading to that attachment bond? Archer sees this as controlled both by a general mechanism, usually involving exposure learning, and by a series of specific responses to cues. These responses steer relationships towards those that will result in enhanced ‘fitness’ — a concept never fully defined here, but apparently meaning reproductive success. It is then the breaking of



Made to mourn: grief is not an illness but a natural reaction to the breaking of bonds of attachment.

attachment bonds which is said to produce grief.

The evolutionary viewpoint is also used to regard primitive grief as a deficit reaction, occurring when a significant other is absent, and leading to searching and protest reactions. In adult humans, these reactions then extend to involve complex mental processes, limiting the amount of distressing information that is received. Conventionally, attempts have been made to overcome this kind of equilibrium by 'grief work', but Archer seriously questions the validity of such an approach.

His writing sets contemporary ideas in both cultural and historical contexts, undertakes an extensive review of research, and looks at such specific issues as the loss of a child, parent or friend (in spite of the preference for evolutionary principles). Like many attempts to move a subject on in one significant bound, it promises rather more than it delivers. But no one with a serious interest in grief can afford to ignore it. □

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A model subject

Global Energy and Water Cycles

edited by K. A. Browning and R. J. Gurney
Cambridge University Press: 1999. 292 pp.
£50, \$95

David Rind

The interactions between energy and water cycles are basic to both weather forecasting and climate-change prediction. This book builds on a 1994 conference of the Global Energy and Water Cycle Experiment and attempts to assess our understanding of this interaction for forecasting on both short and long timescales.

Almost all of the relevant processes involved in the water and energy cycle (with the notable exception of sea ice) have their own subsections in the book. These include such diverse fields as numerical schemes for water-vapour advection, cloud physics, the movement of water through the soil and the ocean's response to fresh water. Each section presents cutting-edge science, emphasizes uncertainties and projects what future research is needed and likely.

The book focuses on how a process works and how it can be modelled, especially in the global general circulation models used to predict climate change. The problems are immense. To model the process, we must first understand it, which means we must be able to observe it. Observations of many hydrological processes are quite poor. Using available data, modellers have provided detailed representations of particular

Science in culture

Surely You're Joking, Mr. Feynman

(Adventures of a Curious Character)

a stage performance by Mike Maran Productions

John Polkinghorne

Richard Feynman is one of a very small band of theoretical physicists whose names have a chance of being recognized by the general public. This is not because of a just appreciation of the powerful calculational techniques that won him a Nobel prize for his work in quantum electrodynamics. Rather, it is because of the persona he created for himself as a fun-loving, bongo-drum-playing guy who just happened to be brilliant at physics. This carefully cultivated image is powerfully conveyed in the best-selling book after which this stage production has been named and from which it draws much of its material.

(Characteristically, the book was compiled from verbal anecdotes by a member of the Leighton family, faithful amanuenses to Feynman, who was himself an actor rather than a playwright.)

Many people have enjoyed the book, though I have to say that, though I am a great admirer of Feynman the physicist, I have never cared for it much. Beneath all the jokiness there is a clear subtext: I am cleverer than anyone else and here are a hundred stories to prove it. But the image of the New York kid was a mask behind which a much more complex and interesting character was concealed. This is evident from the fact that when James Gleick came to write his balanced and successful biography, *Genius*, he found a detailed and carefully preserved archive available to him — not quite what one might have expected of someone whose public stance was that he would have liked to have ducked out of all the trouble involved in accepting a Nobel prize (a story repeated in this show but one that I have never believed).

Both the persona and something of the man beneath it are displayed in this lively stage production. Its form is essentially that of the cabaret, a succession of stories, accompanied by visual images and punctuated by episodes of the South American rhythms Feynman liked so

processes, with the relevant physics often occurring on very small scales. Whether these processes can be accurately simulated in terms of the larger-scale variables used in general circulation models, what level of detail is needed to make global models useful, and how representations can be made consistent among the different processes are three main themes in this book.

While the level of presentation is quite high, there is no attempt to make the coverage systematic. For example, there is no figure showing the climatological global precipitation field, as uncertain as it is.

This book is most appropriate for researchers and advanced graduate students, since it will update them on the diverse com-



much, skilfully performed on a variety of exotic instruments.

There are serious moments — the tragic early death of his first wife Arlene is recalled and there is an account of

Feynman's agonized reaction to the devastation of Hiroshima by the atomic bomb he had helped to build.

Dick Feynman had a deep intuitive understanding of vast ranges of science outside his chosen speciality and a considerable ability to convey insight to non-scientists in a lucid and striking way. In the show, this is best exemplified by his beautiful observation that a tree grows out of the air and not out of the earth, and by his incisive and deceptively simple contribution at the end of his life to the enquiry into the *Challenger* disaster, identifying the cause as the effect of low temperature on the seals of the fuel compartments.

The production drags a little towards the end of its hour-and-a-half, when a valiant attempt to convey something of the Feynman diagram technique is, almost inevitably, somewhat confused and confusing, and where there is, perhaps, undue recourse to drumming to fill in gaps in the continuity.

Nevertheless, Mike Maran is to be congratulated on a lively contribution to what seems to be a growing genre of dramatic performances with some scientific content. I attended a 4 p.m. performance at which at least half the audience appeared to be under the age of 16. I am sure that these young people will have caught something of the excitement and value of science through this performance, inspired by someone who was both a very great scientist and an accomplished showman. □

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ponents involved in climate modelling. But state-of-the-art books in rapidly developing fields have a relatively short shelf-life. Significant developments have already occurred in the three years since most of these articles were written. For example, we have seen the launch of the Tropical Rainfall Measurement Mission and a moisture-conserving form of the semi-Lagrangian scheme for water-vapour advection has been developed. I estimate that another such book will be needed in around five years. With luck, the editors will consider making this a series. □

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Impairment of dynamin's GAP domain stimulates receptor-mediated endocytosis

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Dynamin is a GTP-hydrolysing protein that is an essential participant in clathrin-mediated endocytosis by cells. It self-assembles into 'collars' *in vitro* which also form *in vivo* at the necks of invaginated coated pits. This self-assembly stimulates dynamin's GTPase activity and it has been proposed that dynamin hydrolyses GTP in order to generate the force needed to sever vesicles from the plasma membrane. A mechanism is now described in which self-assembly of dynamin is coordinated by a domain of dynamin with a GTPase-activating function. Unexpectedly, when dynamin mutants defective in self-assembly-stimulated GTPase activity are overexpressed, receptor-mediated endocytosis is accelerated. The results indicate that dynamin, like other members of the GTPase superfamily, functions as a molecular regulator in receptor-mediated endocytosis, rather than as a force-generating GTPase.

Dynamin is essential for receptor-mediated endocytosis and for recycling of synaptic vesicles in neurons. It is believed to play a direct role in detaching clathrin-coated vesicles from the plasma membrane (reviewed in refs 1, 2). Dynamin is a member of a growing subfamily of functionally diverse GTPase proteins (reviewed in ref. 3) which can be distinguished from small and heterotrimeric GTPases by their large size (relative molecular masses of 70K–100K), relatively low affinity for GTP (K_m , 10–100 μ M) and higher intrinsic rates of GTP hydrolysis (k_{cat} , 2–100 min^{-1}). Dynamin serves as the prototype member of this GTPase subfamily.

In its native state, dynamin exists as a homotetramer⁴. When diluted into buffers of low ionic strength, dynamin tetramers spontaneously self-assemble into supramolecular structures con-

sisting of single rings and spirals⁵. Self-assembly *in vitro* stimulates dynamin's GTPase activity 5–10 fold^{6,7}. When dynamin assembly is promoted by an artificial template such as microtubules or acidic phospholipids, GTPase stimulation is >50-fold^{8,9}.

Dynamin spirals have also been observed *in vivo*, appearing as electron-dense 'collars' around the necks of endocytic pits. At the restrictive temperature, *Drosophila* bearing a temperature-sensitive allele of the dynamin orthologue *shibire* accumulate endocytic pits at the synapse, most of which are circled once at their necks by a short dynamin spiral¹⁰. In contrast, long dynamin spirals assemble on permeabilized rat-brain synaptosomes, forming elongated membrane tubules following incubation with GTP- γ S (ref. 11). Dynamin also self-assembles around liposomes containing acidic phospholipids, deforming them into tubules^{12,13}. Strikingly, addition of GTP, but not of its non-hydrolysable analogue GTP- γ S, to dynamin-encircled phosphatidylserine tubules causes their rapid fragmentation into small vesicles¹². However, GTP hydrolysis by dynamin is not sufficient to vesiculate liposomes composed of a more physiological mixture of lipids¹³, and, consistent with this, dynamin is not sufficient to drive endocytic coated-vesicle formation in perforated cells (reviewed in ref. 1).

It has been proposed that GTP hydrolysis by assembled dynamin drives a concerted conformational change that is necessary, but not sufficient, for endocytic coated-vesicle formation^{5,14}. Others have suggested that dynamin functions as a molecular 'pinchase' that alone drives membrane fission^{15,16}. Many features of these models remain to be tested *in vivo*, particularly the role of dynamin self-assembly and assembly-stimulated GTP hydrolysis in endocytosis.

GED regulates GTPase activity

We first investigated the mechanism of dynamin assembly and its stimulation of GTPase activity by focusing on a domain of dynamin named GED (for GTPase effector domain)⁴ (Fig. 1a). Crosslinking studies established that this region is closely associated with the N-terminal GTPase domain, and removal of GED by limited proteolysis gave a truncated form of dynamin that could bind GTP but had significantly impaired GTPase activity⁴. To determine whether GED functioned as a GTPase-activating protein (GAP) or as a guanine-nucleotide-exchange factor (GEF), we expressed the GTPase domain and GED independently as fusion proteins with glutathione S-transferase (GST). Unexpectedly, based on our previous interpretation⁴, the isolated GTPase domain hydrolysed GTP

Table 1 Kinetic parameters of basal and stimulated dynamic GTPase activity

Enzyme $\pm$ additions	Kinetic parameters	Properties	
Full-length dynamin		Cooperative?	AlF ₄ ⁻ sensitive
Dyn(WT) (unassembled)	$k_{cat} = 2.5 \pm 0.2 \text{ min}^{-1}$	No	No
+GED(WT)	$k_{cat} = 120 \pm 10 \text{ min}^{-1}$ $K_M(\text{GED}) = 3 \pm 0.5 \mu\text{M}$ Hill constant = 2.8	Yes	Yes
+GED(K694A)	$k_{cat} = 123 \pm 5 \text{ min}^{-1}$ $K_M(\text{GED}) = 10 \pm 1 \mu\text{M}$	No	Not tested
+microtubules	$k_{cat} = 165 \pm 10 \text{ min}^{-1}$	Yes ⁸	Yes ¹⁷
Dyn(R725A) (unassembled)	$k_{cat} = 2.3 \pm 0.2 \text{ min}^{-1}$	No	No
+GED(WT)	$k_{cat} = 20 \pm 5 \text{ min}^{-1}$	Not tested	Yes
+microtubules	$k_{cat} = 22 \pm 2 \text{ min}^{-1}$	Yes	Not tested
Dyn(K694A) (unassembled)	$k_{cat} = 2 \pm 0.1 \text{ min}^{-1}$	No	No
+GED(WT)	$k_{cat} = 120 \pm 5 \text{ min}^{-1}$ $K_M(\text{GED}) = 10 \pm 0.8 \mu\text{M}$	No	Not tested
+GED(K694A)	$k_{cat} = 123 \pm 7 \text{ min}^{-1}$ $K_M(\text{GED}) = 20 \pm 1 \mu\text{M}$	No	Not tested
+microtubules	$k_{cat} = 123 \pm 10 \text{ min}^{-1}$	Yes	Not tested
GTPase domain			
GST-GTPase	$k_{cat} = 2.1 \pm 0.2 \text{ min}^{-1}$	No	No
+GED(WT)	$k_{cat} = 10 \pm 2 \text{ min}^{-1}$ $K_M(\text{GED}) = 5 \pm 0.5 \mu\text{M}$ Hill constant = 1.8	Yes	Not tested

* Theoretically determined by Eadie-Hofstee calculations³³.

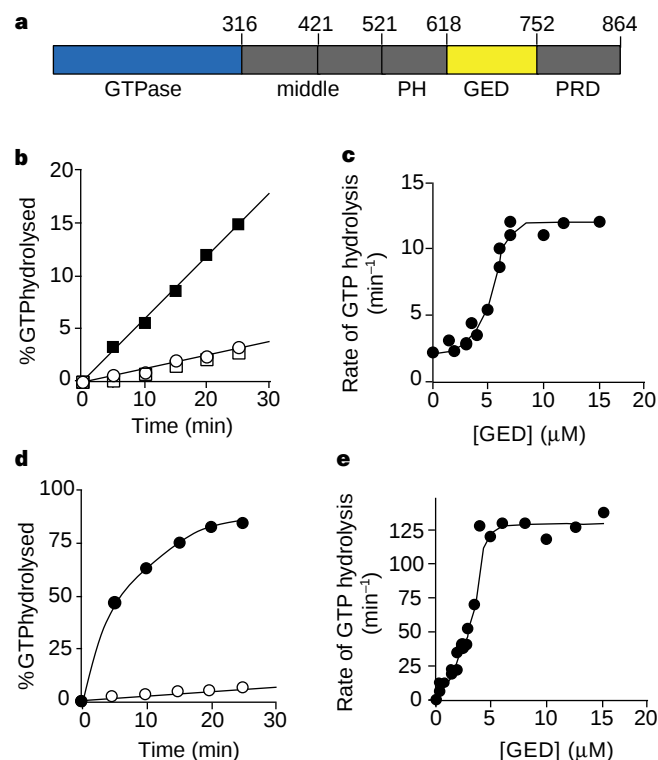


Figure 1 GED stimulates both the minimal GTPase domain and full-length dynamin. **a**, Domain structure of dynamin showing the N-terminal GTPase domain, the PH (pleckstrin-homology domain), the GED (GTPase effector domain) and the C-terminal PRD (proline/arginine-rich domain). **b**, Time course for GTP hydrolysis by 0.15 μ M dynamin tetramers (○), 0.15 μ M GST-GTPase domain alone (□) or with 10 μ M GED (■). **c**, The concentration dependence of GED-stimulated GTPase activity of 0.15 μ M GST-GTPase. **d**, Time course for GTP hydrolysis of full-length dynamin incubated with (●) or without (○) 5 μ M GED. **e**, Concentration dependence of GED-stimulated GTPase activity of 0.15 μ M dynamin tetramers. The Hill constant and K_M were calculated as described in methods.

at the same intrinsic rate as unassembled dynamin tetramers (Fig. 1b and Table 1).

Although GED did not contribute to the basal rate of GTP hydrolysis, addition of GED, isolated after cleavage from GST, stimulated GTP hydrolysis by the GTPase domain 6-fold (Fig. 1b). The concentration dependence for this activation was sigmoidal (Hill constant, 1.8), which is indicative of cooperative binding of GED to the GTPase domain (Fig. 1c and Table 1). Thus, more than one GED is required to stimulate GTPase activity, suggesting that this interaction mimics dynamin assembly and assembly-stimulated GTP hydrolysis. We therefore investigated whether GED alone could mediate assembly-stimulated activation when added to full-length dynamin and found that addition of recombinant GED to unassembled dynamin tetramers resulted in a striking, greater than 50-fold stimulation of GTPase activity (Fig. 1d). This activation by GED was comparable to the highest rates achieved during the promotion of dynamin self-assembly by microtubules^{17,18}. Moreover, this GED stimulation of dynamin was highly cooperative (Fig. 1e and Table 1), with a Hill constant consistent with maximal stimulation requiring the interaction of four GED molecules with a single dynamin tetramer. Addition of GED did not further stimulate previously assembled dynamin (data not shown), suggesting that GED functions by mimicking the self-assembly reaction.

Our results indicate that GED may directly mediate dynamin's assembly-stimulated GTPase activity, in which case it should inhibit self-assembly of dynamin tetramers. To this, we used an assay in which the assembly of dynamin tetramers into rings and spirals at

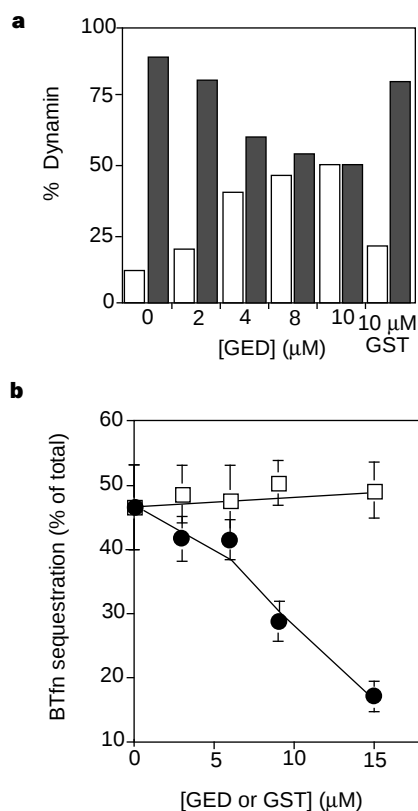


Figure 2 GED inhibits dynamin self-assembly and receptor-mediated endocytosis. **a**, Dynamin was incubated with increasing concentrations of GED or GST for 30 min on ice in HCB52 and then diluted 10-fold into HCB0 to induce self-assembly. Self-assembly reactions were kept at room temperature for 10 min before separation into supernatant (white bars) and pellet (shaded bars) fractions by ultracentrifugation. **b**, Endocytosis of biotinylated transferrin (BTfn) in perforated A431 cells^{19,20}. The cytosol was incubated with increasing concentrations of GST (□) or GED (●) for 30 min on ice before addition to perforated A431 cells. The amount of internalized BTfn after 15 min at 37°C was determined by inaccessibility to avidin.

low ionic strength was measured by centrifugation^{5,6} and found that GED inhibited the assembly of intact dynamin in a concentration-dependent manner (Fig. 2a), whereas GST had no effect. We conclude that GED has a dual function: as an assembly domain and, after assembly, as a specific activator of GTP hydrolysis.

GED inhibits receptor-mediated endocytosis

Our results show that isolated GED stimulates GTP hydrolysis by dynamin but inhibits dynamin self-assembly. We next tested the effect of GED on dynamin function in receptor-mediated endocytosis by using a perforated-cell assay that reconstitutes early and late events in endocytic clathrin-coated vesicle formation^{1,19,20}. Perforated A431 cells were incubated with biotinylated transferrin (BTfn), an ATP-regenerating system, a cytosolic fraction derived from K562 cells, and increasing concentrations of GED or GST as a control. We assessed the extent of sequestration of BTfn into constricted coated pits and coated vesicles by its acquired inaccessibility to externally added avidin and found that GED potently inhibited the receptor-mediated sequestration of BTfn in a concentration-dependent manner (Fig. 2b), whereas GST had no effect. Thus, GED is active in the physiological context of endocytic coated-vesicle formation. Its inhibitory effects could reflect inhibition of dynamin self-assembly and/or GED's ability to stimulate GTP hydrolysis in the absence of dynamin self-assembly. The latter activity would shift dynamin to its GDP-bound state. Indeed,

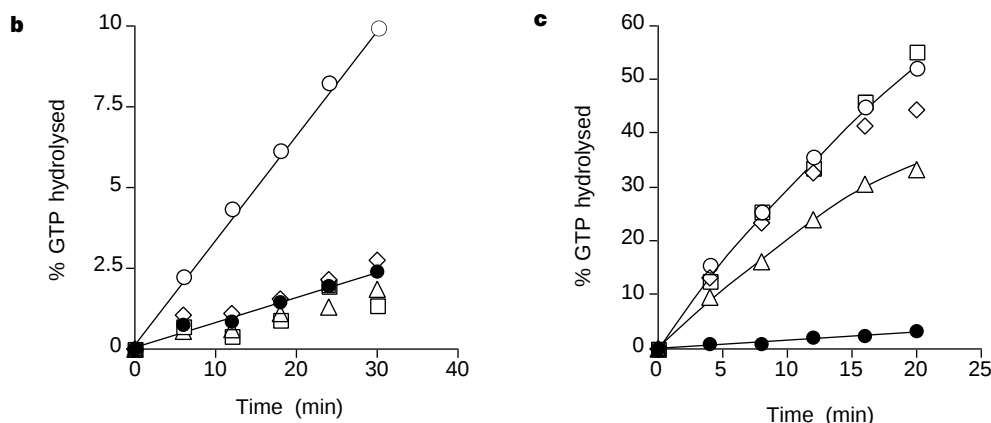


Figure 3 Identification of active residues within GED. **a**, Sequence alignment of GEDs from dynamin family members: Dyn, human dynamin-1 (aa 657–744)³⁴; Shi, *shibire*, the *D. melanogaster* orthologue of dynamin (aa 654–742)^{35,36}; Vps1, a *S. cerevisiae* vacuolar sorting protein (aa 616–704)³⁷; Dnm1 (ref. 38), a *S. cerevisiae* protein involved in mitochondrial structure maintenance (aa 642–723)³⁹; MxA, a human interferon-inducible antiviral protein (aa 503–591)⁴⁰; and Phr, *A. Thaliana* phragmoplastin (aa 521–613), which is associated with cell-plate formation during cell division⁴¹. Identity is indicated by black boxes, similarity by grey boxes.

Alignment was performed using the BCM Search Launcher. Numbers above the Dyn1 GED sequence indicate Lys and Arg residues mutated to Ala and tested for their GAP activity on GST–GTPase and full-length dynamin. **b, c**, Stimulation of GST–GTPase (**b**) and full-length dynamin (**c**) by wild-type and mutant GEDs. The time course is shown of GTP hydrolysis by 0.15 μ M enzyme alone (●) or incubated with 10 μ M of either GED(WT) (○), GED(R725A) (□), GED(K694A) (Δ), or GED(R730A) (◇).

receptor-mediated endocytosis in perforated cells is inhibited by GDP- β S (ref. 20).

GED is an assembly-dependent GAP

Most GTPases are regulated by accessory factors that act either as GAPs or as GEFs. Our preliminary results indicated that the GED domain probably does not function as a GEF. We were unable to isolate a stable dynamin–GDP complex, consistent with dynamin's known low affinity for GDP^{6,8,21}, and the rate of Mant–GDP release, as measured by fluorimetry, was much faster than the activated rate of GTP hydrolysis (results not shown). Other results indicated that GED may function as a GAP. Inhibition by AlF_4^- is diagnostic of GAP-stimulated GTP hydrolysis²². Although AlF_4^- promotes dynamin self-assembly²³, we found that it potently inhibited the assembly-stimulated GTPase activity of dynamin, but had no effect on dynamin's basal rate of GTP hydrolysis (Table 1). GED is highly conserved among dynamin family members (Fig. 3a), which suggests that they all encode an intramolecular GAP.

To identify the residues in GED that are involved in assembly-stimulated GTP hydrolysis, we considered the known role of arginine and lysine residues in rasGAP function²⁴. Each arginine and lysine residue in GED (Fig. 3a) was mutated to alanine and the mutant GEDs were expressed and purified. These were individually tested for their ability to stimulate the GTPase domain and full-length dynamin: most mutations were without effect, but substitution of K694, R725 or R730 with alanine blocked the stimulation of the GTPase domain by GED (Fig. 3b). By contrast, at the concentrations tested, each of these mutant GEDs stimulated dynamin tetramers (Fig. 3c). The main difference between these two assays is that full-length dynamin contains its own wild-type GED (GED_{cis}) and its activity is stimulated by exogenous GED (GED_{trans}). The ability of mutant GEDs to stimulate dynamin tetramers *in trans*

suggests that the active residues are not provided by GED_{trans}. Stimulation of isolated GTPase domains, however, requires a functional GED molecule acting *in cis* and a second functional GED acting *in trans*. In this assay, both GEDs carry the same mutation. Our results indicate that GED_{cis} supplies catalytic residues and that GED_{trans} activates GED_{cis} upon assembly.

GED mutants impair stimulated GTPase activity

If this model is correct, then when these mutations are placed in full-length dynamin they should selectively inhibit assembly-stimulated GTPase activity. We found that, in comparison with wild-type dynamin (dyn(WT)), dyn(R725A) was poorly stimulated by GED (Table 1), and had significantly reduced assembly-stimulated GTPase activity even in the presence of microtubules (Fig. 4a). The basal rate of hydrolysis for dyn(R725A) in the absence of microtubules or GED was the same as for dyn(WT) (Table 1), further supporting our hypothesis that in the unassembled state, GED_{cis} does not contribute to basal GTPase activity. These data unambiguously establish GED as being an intramolecular GAP and R725 as being an essential catalytic residue.

Surprisingly, K694 does not seem to be directly involved in catalysis. Instead, Fig. 4b shows that the K694A mutation affects the affinity of exogenous GED for dynamin tetramers. The extent to which GED(K694A) stimulated dyn(WT) (Fig. 4b, circles) was the same as that to which GED(WT) stimulated dyn(K694A) (Fig. 4b, triangles). However, the apparent affinity of dynamin for GED under these conditions was ~3-fold lower (Table 1) than when both components were wild-type (Fig. 4b, dashed line). When both full-length dynamin and exogenous GED carry the K694A mutation (Fig. 4b, squares), the apparent affinity for GED was even less (~7-fold lower; Table 1). The effect of the K694A mutation can be overcome by incubation at low salt in the presence of microtubules,

which serve as a template for self-assembly. Under these conditions, the stimulated rate of GTP hydrolysis by dyn(K694A) approached that of wild type (Fig. 4a), in agreement with the calculated maximal rate of GTP hydrolysis in the presence of GED (Table 1). Together, these results indicate that GED_{trans} interacts with dynamin tetramers through their GED_{cis} domains and that assembly-stimulated GTPase activity is mediated by GED_{cis}-GED_{trans} interactions.

GED-GTPase and GED-GED interactions were previously detected by crosslinking of dynamin⁴, and occur here between isolated GED and GST-GED or GST-GTPase, but not with GST alone (Fig. 4c). Both mutant GEDs were indistinguishable from wild type with respect to their ability to interact with GST-GTPase (Fig. 4c, lanes 1–3), in agreement with the formation of a stable complex between GED and GTPase domains after limited proteolysis⁴. Consistent with the differences in affinity measured by GTPase stimulation (Table 1), GED(K694A) had a 5-fold reduced ability to form complexes with GST-GED(K694A) (Fig. 4c, lane 6) compared to pairs of wild-type (Fig. 4c, lane 4) or R725A-mutant (Fig. 4c, lane 5) GEDs.

Our results indicate that the principal defect in dyn(K694A) is a reduced ability to self-assemble, which we confirmed by sedimentation assay: as expected, dyn(K694A) has a significantly reduced tendency to self-assemble, whereas dyn(R725A) assembles as well as wild-type dynamin (Fig. 4d). We conclude that GED is a self-assembly domain and an assembly-dependent intramolecular GAP.

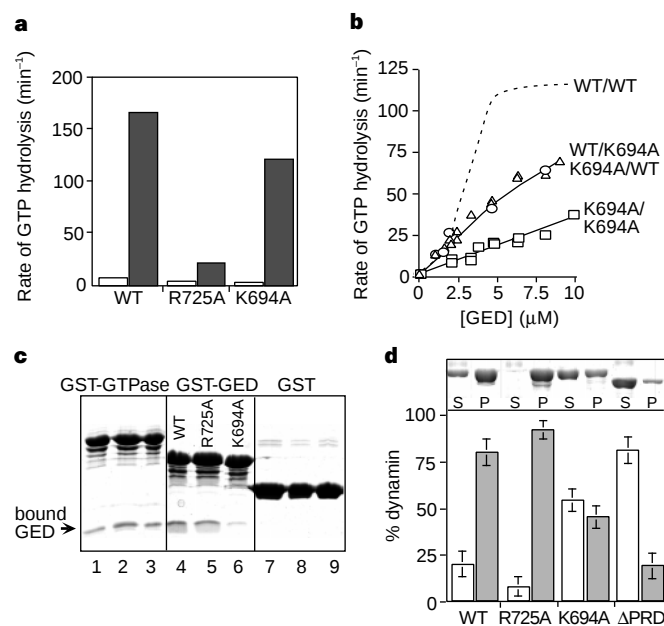


Figure 4 GTPase and self-assembly activities of full-length wild-type, R725A and K694A mutant dynamin. **a**, Rate of GTP hydrolysis of 1 μ M dyn(WT), dyn(R725A) or dyn(K694A) assayed in HCB10 in the absence (white bars) or presence (shaded bars) of 2 μ g of microtubules. **b**, Concentration dependence of stimulation of full-length dyn(WT) by GED(K694A) (○); of full-length dyn(K694A) by GED(WT) (Δ), or of full-length dyn(K694A) by GED(K694A) (□). Dashed line, data for dyn(WT) stimulated by GED(WT), redrawn from Fig. 1e. GTPase activity of full-length dynamin (0.2 μ M) was assayed in the presence of increasing concentrations of GED ($n = 4$). **c**, Direct interactions of isolated GED with GST-GED and GST-GTPase domain. Coomassie-blue-stained gel of: lanes 1, 4, 7, isolated GED(WT); lanes 2, 5, 8, GED(R725A); lanes 3, 6, 9, GED(K694A), coprecipitated with: lanes 1–3, GST-GTPase; lane 4, GST-GED(WT); lane 5, GST-GED(R725A); lane 6, GED(K694A); lanes 7–9, GST bound to glutathione beads. **d**, Assembly activity of 1.5 μ g dyn(WT), dyn(R725A), dyn(K694A) and dyn(Δ PRD) assayed as for Fig. 2b. The Δ PRD truncation mutant is defective in self-assembly⁶. The histogram shows average $\pm$ s.d. ($n = 3$). Inset, Coomassie-blue-stained gel from a single representative assay (S, supernatant; P, pellet).

We propose that K694 is situated at the interface between two GEDs, where it stabilizes their interaction during dynamin self-assembly. GED-GED interactions are needed to position the active residue, R725, to mediate the stimulation of GTP hydrolysis.

Role of dynamin in endocytosis

Our biochemical analysis of GED function enabled us to design a novel class of dynamin mutants that maintain their basal rate of GTPase activity but whose stimulated activity is greatly impaired, either because they have lost a catalytic residue (dyn(R725A)), or their propensity to self-assemble (dyn(K694A)). We can use these mutants to investigate the role of dynamin assembly and assembly-stimulated GTPase activity *in vivo*. If, as current models for dynamin function predict, assembly-stimulated GTPase rates are essential for receptor-mediated endocytosis, then overexpression of these mutants should inhibit the process in intact cells, but we found the opposite to be true. BHK cells were co-transfected with wild-type or mutant dynamin constructs, together with a construct encoding the human transferrin receptor in order to measure endocytosis of transferrin preferentially in the transfected population of cells. Overexpression of wild-type dynamin had no effect on endocytosis relative to mock-transfected controls (results not shown), whereas overexpression of a dominant-negative mutant of dynamin (S45N) inhibited receptor-mediated endocytosis of transferrin, as expected²⁵ (Fig. 5). In contrast, overexpression of dyn(R725A) or dyn(K694A) caused a significant stimulation of the rate of endocytosis. In all experiments ($n = 5$), the extent of stimulation compared to cells overexpressing wild-type dynamin was between 1.6- and 1.8-fold. The same relative increase in endocytosis was observed under conditions that measured only a single round of BTfn uptake, whereas the recycling of transferrin receptors was not affected (data not shown). Taken together, these results show that overexpression of dynamin mutants selectively affected an early stage in receptor-mediated endocytosis. The specific defect in assembly-dependent GAP activity of the R725A

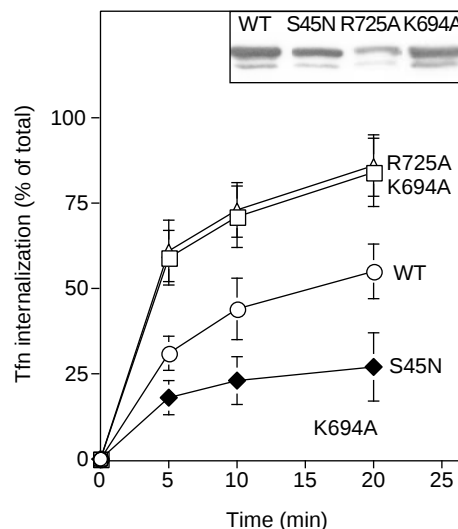


Figure 5 Receptor-mediated endocytosis is regulated by dynamin-GTP. BHK cells were co-transfected with cDNA encoding the human transferrin (TfR) receptor and either dyn(WT) (○), dyn(S45N) (◆), dyn(R725A) (Δ), or dyn(K694A) (□) and cultured for an additional 24 h before collecting the cells and assessing the rate of BTfn internalization (see Methods). Results are average $\pm$ s.d. of 5 independent experiments. Inset, expression of dyn(WT), dyn(S45N), dyn(K694A) and dyn(R725A) is comparable, as shown by western-blot analysis using rabbit antiserum raised against GED. On average, 50–60% of the cells were transfected and the overexpression of dynamin was ≥ 10 -fold over endogenous. The smaller band corresponds to C-terminally proteolysed dynamin.

and K694A mutants will prolong the GTP-bound state of dynamin, so dynamin-GTP must control a rate-limiting step in endocytosis.

Discussion

We have reported a previously unknown molecular mechanism employed by dynamin to regulate its GTPase cycle. Dynamin contains an intramolecular GAP which is silent in the unassembled state and is activated through interaction with a second GAP upon assembly of dynamin tetramers into higher-order structures. The arginine residue R725 participates directly in catalysis and is invariant in the dynamin subfamily; the lysine residue K694 is involved in self-assembly and is highly conserved (Fig. 3a). Moreover, analysis of the distantly related Mx proteins supports the idea that assembly-dependent GTPase activation is a common feature of all dynamin-family members. Specifically, carboxy-terminal deletions in the human MxA GED inhibit GTPase activity^{26,27}. Together, these results suggest that the mechanism of GED's assembly-dependent GAP activity serves as a new framework for GTPase regulation in this family.

Contrary to predictions based on recent models for dynamin function^{14–16}, we find that endocytosis was accelerated in cells transiently overexpressing dynamin mutants that were defective in assembly-stimulated GTP hydrolysis. On the basis of this unexpected result, we propose a new model for dynamin's GTPase cycle in endocytosis (Fig. 6). Dynamin-GTP, like other GTPases, is the 'active' conformation that recruits downstream partner(s) as components of the fission machinery. Dynamin assembly is required to activate its intramolecular GAP and thus inactivate the GTPase. We suggest that GAP activation occurs through stacking interactions between dynamin tetramers which can form only on completion of

an overlapping turn of the dynamin spiral: it would be difficult for collar assembly to occur if the lateral interactions between tetramers were sufficient to activate the GAP. Thus the dynamin GTPase collar could act as a sensor that measures the dimensions of the neck to monitor the progress of the fission reaction. Overexpression of a dynamin mutant with impaired self-assembly (dyn(K694A)) also accelerates endocytosis, suggesting that efficient assembly of the collar is not a prerequisite to vesicle budding. GTP hydrolysis converts the enzyme to the 'inactive' GDP-bound form and triggers disassembly of the collar, releasing dynamin and the fission machinery for further rounds of vesicle formation. We conclude that dynamin functions as a molecular regulator, like a classical GTPase.

This new model for dynamin function is consistent with several observations. (1) GTP, but not GTP- γ S, causes the release of dynamin from microtubules²¹ and the disassembly of preassembled dynamin²³; (2) according to our model, the rate of GTP hydrolysis will depend on the ratio of internal rings to spiral ends: in fact, maximal rates of GTP hydrolysis are achieved when dynamin forms long spirals on artificial templates^{8,28}; (3) dynamin and GTP- γ S are sufficient to drive the detachment of preformed coated pits from isolated plasma-membrane preparations in a cell-free assay measuring the very late stages of coated vesicle formation²⁹; (4) the regulation of dynamin function at the synapse by phosphorylation/dephosphorylation prompted the suggestion that dynamin-GTP is the active form of the enzyme¹⁵: dynamin is phosphorylated in the resting neuron *in vitro*, which increases its GTPase activity—upon depolarization at the synapse, dynamin is rapidly dephosphorylated, so activation of endocytosis at the synapse correlates with deactivation of dynamin GTPase¹⁵.

Our model is apparently inconsistent with observations that GTP- γ S blocks endocytosis in perforated cells²⁰ and leads to an accumulation of long dynamin spirals on endocytic intermediates¹¹. The aberrant assembly of dynamin that occurs in the presence of GTP- γ S may inhibit vesicle detachment either by recruitment of the fission machinery to diffuse sites along the neck, or by sterically impeding the fission reaction. Dynamin-GDP may also be essential in the early stages of endocytic coated-vesicle formation for recruiting upstream factors. Finally, these other GTP analogues could be affecting other GTPases involved in endocytosis.

If, as we suggest, dynamin is the master regulator of endocytosis, then its reported interactions with signalling molecules such as Grb2 (ref. 30), c-Src (ref. 31), G-protein $\beta\gamma$ subunits⁷ and the MAP kinase Erk2 (ref. 32) may provide a mechanism by which receptor-mediated endocytosis is regulated by external signals. Identification of the downstream partners that interact specifically with dynamin-GTP will be prerequisite to understanding the mechanism of endocytosis. □

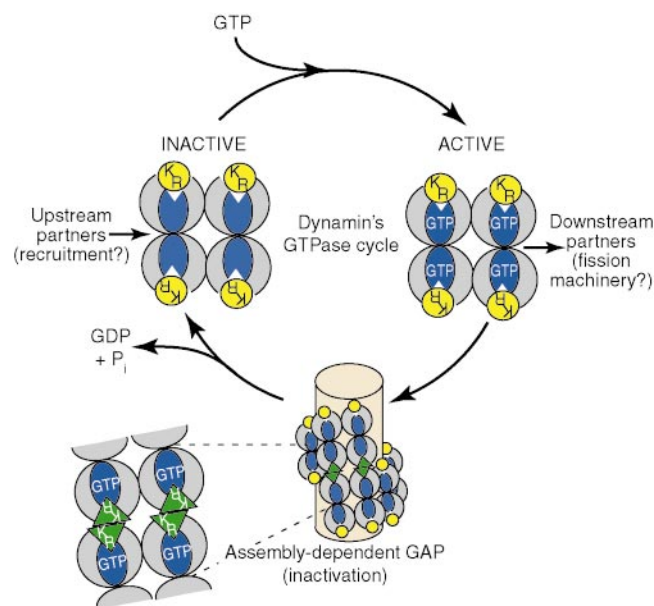


Figure 6 An intramolecular, assembly-dependent GAP functions to inactivate dynamin. Like other GTPases, dynamin-GTP is in the 'active' conformation and recruits the fission machinery required for vesicle formation. Its intrinsic GAP, GED (yellow circles), is silent in the unassembled state. Upon assembly, GED-GED interactions, mediated in part by K694, result in conformational changes that activate the GAP. These allosteric interactions position residues (such as R725) correctly within GED at the active site so that they can participate in catalysis. We propose that GED activation (green triangles) occurs only at the interface between stacked dynamin tetramers. In this way, the dynamin collar monitors the dimensions of the neck and can serve as a geometric sensor that regulates endocytosis. In this model, dynamin self-assembly functions to activate its GAP and not to drive fission *per se*. GTP hydrolysis by dynamin signals the completion of the fission reaction and returns dynamin to its inactive state.

Methods

Preparations of cDNA constructs. The GTPase domain was amplified using the Expand Long Template PCR system (Boehringer Mannheim) and pFASTBac-dynamin^{wt} as a template to generate a 961-bp fragment encoding the first 316 amino acids of dynamin. Primers used to generate the DNA fragment encoding the GTPase domain were 5'-AC TAG TGG ATC CAA GGA GCC GCC GCC ATG-3' (*Bam*HI site underlined) and 5'-CC GAA TTC ATC AGG GCG CTA GTT CTT GTA-3' (mutations introducing a stop codon instead of Phe are in bold; the *Eco*RI site is underlined). After digestion with *Bam*HI and *Eco*RI, the fragment was recloned into pGEX-Xa for expression in *E. coli*.

The GED expression vector was constructed by recloning the 1.4-kb *Stu*I-*Hpa*I fragment from pTM1- Δ PRDdynamin⁶ into the *Sma*I site in pGEX-4T3. The pGEX-GED construct obtained encodes amino acids 618 to 752.

Mutations in GED were constructed using standard two-step PCR mutagenesis with *Pfu* (Boehringer Mannheim) and primers corresponding to the 5' and 3' pGEX sequences from the *Sma*I site, and 23-mers corresponding to coding and non-coding sequences, with the mutation (Ala instead of Arg or Lys) in the middle. After the second round of PCR, fragments were cloned into

the pCR2.1 vector (Invitrogen) and sequenced by using M13 primers. Positive clones were recloned as *Bam*HI–*Xho*I fragments into the pGEX-4T3 vector and used for expression.

pFASTBac–dynamin harbouring R725A and K694A mutations were made by two-step PCR mutagenesis using primers 5′-CGG CAG CTG GAG CTA GCC-3′ (for 5′) and 5′-C CCA A GC TTA GAG GTC GAA GGG GGG CCT GGG GCT CTC-3′ (for 3′; *Hind*III site underlined), the mutagenic primers were as described above for GED. After the second round of PCR, fragments (540 bp) were cloned into the pCR2.1 vector (Invitrogen) and sequenced by using M13 primers. Positive clones were cut with *Nsi*I–*Hind*III to generate a 370-bp fragment, which was isolated from an LMP–agarose gel and exchanged for the fragment generated from pFASTBac–dynamin(WT) with the same restriction enzymes. The composition of all constructs was confirmed by sequencing.

Purification of proteins. Wild-type and mutant dynamins were expressed in Tn5 cells and purified as described⁴. Wild-type and mutant dynamin molecules co-eluted by gel-filtration chromatography, indicating that mutants were tetramers. GST–GTPase and GST–GED were expressed in *E. coli* BL21 cells by using pGEX-Xa and pGEX-4T3 vectors respectively (Pharmacia) and standard procedures. GED was obtained by thrombin cleavage of GST–GED in PBS for 6 h at 4°C. Isolated GED was soluble and, based on its circular dichroism spectrum, predominantly α -helical (not shown). All proteins, including GED, were dialysed overnight at 4°C into HCB150 buffer (20 mM HEPES, 2 mM MgCl₂, 1 mM EGTA, pH 7.0, 150 mM NaCl). Aggregates formed during dialysis were removed by centrifugation at 8,000g for 5 min in a microfuge.

Assembly and GTPase assays. Dynamin self-assembly was performed essentially as described⁶, except that the final volume was 100 μ l and contained 5 mM NaCl. After high-speed centrifugation, supernatants were precipitated with 10% trichloroacetic acid and the total protein from the pellets and supernatants was analysed by SDS–PAGE. GTPase was assayed at 37°C as described⁶, except that the assay was done in HCB buffer with 52 mM NaCl, 1 mM DTT, 0.1% BSA and 0.5 mM GTP. For GED stimulation, dynamin was preincubated with GED for 30 min on ice before initiation of the reaction by addition of [α -³²P]GTP. Rates of GTP hydrolysis were calculated from a minimum of five time points and expressed as the percentage of GDP/GTP + GDP. The Hill constant was calculated using the equation $\log[v/(V_{\max} - v)] = h \log[\text{GED}] - \log K$ (ref. 33), and the K_M for GED was calculated using an Eadie–Hofstee plot.

GST–GTPase and GST–GED interactions. GST–GED or GST–GTPase (10 μ g) were bound to glutathione beads and incubated with isolated GED (10 μ g) in 50 μ l HCB buffer containing 52 mM NaCl, 0.1 mM DTT, for 1 h at 4°C with rotation. Beads were collected by centrifugation, washed with 200 μ l of the same buffer, then resuspended in 20 μ l sample buffer and analysed by SDS–PAGE; proteins were detected by Coomassie staining.

Transfection procedure. 5×10^5 BHK cells, maintained in DMEM (GIBCO BRL) were plated on 60-mm dishes 12 h before transfection. Cells were transfected using Lipofectamine Plus reagent (Life Technologies) with 0.75 μ g transferrin receptor cDNA (a gift from C. Enns), together with 4 μ g pcDNA vectors (Invitrogen) encoding wild-type or mutant dynamins of β -galactosidase, according to manufacturers' instructions. All DNA concentrations were adjusted to 1 μ g μ l⁻¹ to maintain constant volumes between reactions. Cells were collected 24–28 h after transfection for internalization assays, as described³⁴.

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A dusty pinwheel nebula around the massive star WR104

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Wolf-Rayet (WR) stars are luminous, massive blue stars thought to be the immediate precursors to some supernovae. The existence of dust shells around such stars has been enigmatic since their discovery about 30 years ago, as the intense ultraviolet radiation from the star should be inimical to dust survival¹. Although dust creation models, including those involving interacting stellar winds², have been put forward to explain these dust shells, the high-resolution observations needed to distinguish between the models have hitherto been lacking. Here we present images of the dust outflow around WR104, obtained using a technique that allows us to resolve detail on scales of about 40 AU at the distance of the star. Our images—taken at two epochs—show that the dust forms a spatially confined stream that follows precisely a linear (or archimedean) spiral trajectory with a rotation period of 220 ± 30 days. These results prove that, in this case, a binary

companion is responsible for the creation of the circumstellar dust. Moreover, the spiral plume makes WR104 the prototype of a new class of circumstellar nebulae, which are unique to systems with interacting winds.

Observations of WR104 were made with the Keck I telescope on 14 April and 4 June 1998, and employed the technique of aperture masking interferometry in order to recover information out to the diffraction limit of the 10-m Keck aperture^{3,4}. We present, in Fig. 1, reconstructed images taken at wavelengths of 1.65 and 2.27 μm (filter bandwidths $\Delta\lambda = 0.33$ and 0.16 μm , respectively) for both observing epochs. As infrared emission from the hot circumstellar dust dominates the infrared region of the spectrum, we may interpret the highly asymmetric curved plumes evident in the maps as tracing the distribution of this material. Previous high-resolution efforts have been restricted to partially resolved one-dimensional visibility curves interpreted in the context of spherically symmetric outflow models^{5,6}. As a comparison with these earlier results, we have fitted a uniform-disk model to our visibilities, azimuthally averaged and cropped to the resolutions then obtained, finding perfect agreement with the 130-mas-diameter disk reported in 1981⁵. This similarity over a timescale of decades is in accord with the inclusion of WR104 in the small handful of 'persistent' dust-producing WRs⁷. Additional interferometric observations at 3.08 μm ($\Delta\lambda = 0.1 \mu\text{m}$) show no evidence of the marked enlargement towards longer wavelengths reported by Dyck *et al.*⁶. However, as is apparent from Fig. 1, the images do not show even remote similarity to a uniform disk, and we hereafter abandon further consideration of circularly symmetric models.

The maps of Fig. 1 consist of two components; a bright central

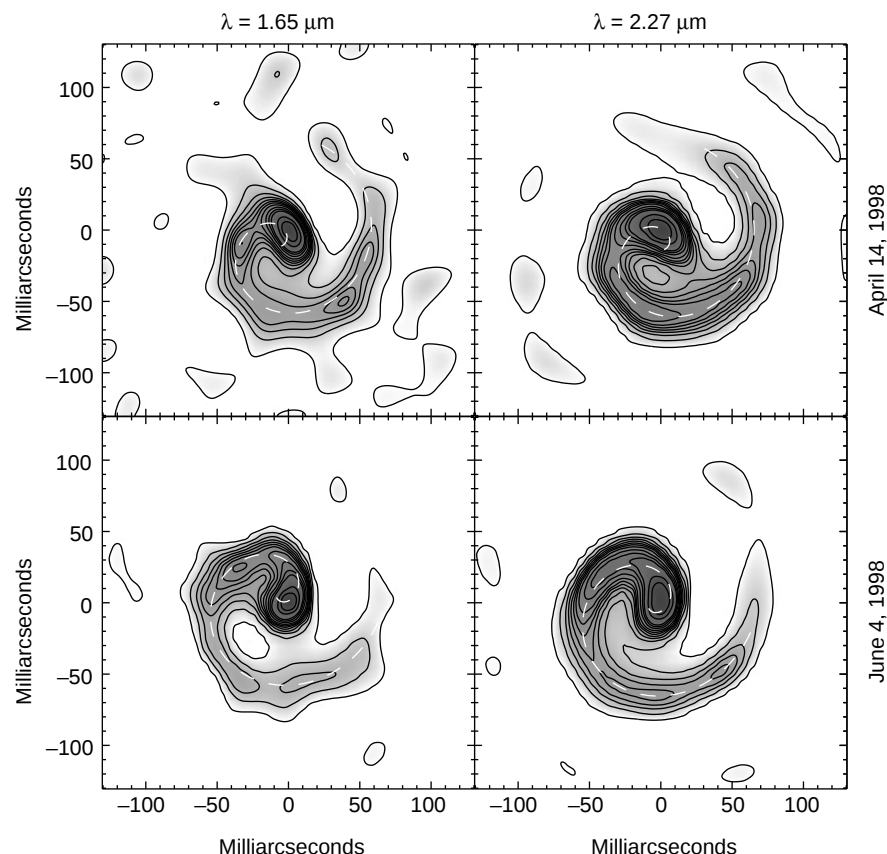


Figure 1 Maps of WR104. Maximum-entropy image reconstructions of WR104 at 1.65 μm (left) and 2.27 μm (right) taken at two separate epochs: April (upper) and June (lower) 1998 (JD 2450918 and JD 2450969, respectively). Contour levels are 0.5, 2, 4, 6, 8, 10, 12, 15, 20, 25, 30, 50 and 70% of the peak. Overplotted on each map is the best-fit archimedean spiral model (dashed line). Observations utilized an annulus-shaped pupil mask to form the interference pattern, which was recorded on a fast-readout (130-ms) infrared array and subsequently processed to extract

Fourier amplitudes and closure phases. Bispectral information constituting ~ 700 baselines and $\sim 7,000$ closing triangles enabled high-quality images to be produced from an algorithm based on the maximum-entropy method^{21,22}. Although these images are at the heart of our discussion, we note that clear and systematic signals betraying the presence of the final image morphology are directly visible in the calibrated Fourier data themselves.

core which appears elongated, and a curved tail which seems to emerge from one end of the elongation. This spiral structure dominates the morphology at both wavelengths employed, and maps taken in April and June 1998 show a high degree of similarity with the striking exception of a clear rotation of the image. The hypothesis of dust formation mediated by the orbital motion of a companion star and subsequent outflow with the stellar wind unifies the spiral structure and the -83° rotation apparent between our two epochs into a simple, elegant geometry. A diagram of our model is shown in Fig. 2, showing the WR with a spectral type OB companion, the dust-formation zone associated with the collision front between the stellar winds², and the resultant curved outflow plume as this dust 'nursery' is carried with the orbital motion. Although the idea of a binary nature for WR104 is not new, it is only within the past few years that the presence of an OB companion was confirmed from detection of hydrogen Balmer absorption features and optical emission-line dilution^{8,9}.

In Fig. 1 we have overplotted the results of fitting a simple geometrical model, consisting of an archimedean spiral where the free parameters are the winding rate and the viewing angle to the observer. These modelling results were obtained by finding a global best fit to all four maps simultaneously, but allowing for a rotation of the spiral structure about the model-derived axis between the two epochs. Implicit in the assumption of an archimedean spiral model is the hypothesis that the material in the spiral is moving out at a uniform velocity, and that new material feeding into the flow insertion point does so at a uniform angular velocity. Although such a model contains the fewest free parameters and yet gives excellent fits to our data, it is important to note that a more complex model may be required if the plume is in a zone where it is being accelerated, or if the orbit of the companion presumed to be mediating the flow is eccentric (for example, ref. 7).

The physical geometry of the system, as derived directly from our model, is a spiral plume rotating with a period of 220 ± 30 days, viewed at an angle of $20 \pm 5^\circ$ from the pole, and with an outflow velocity of $111 \pm 17 \text{ mas yr}^{-1}$ in the plane of the orbit. If we identify this rotation as the orbital period of a binary stellar system, then assuming a combined mass in the range of $20\text{--}50 M_\odot$ (ref. 10) results in a separation of $1.9\text{--}2.6 \text{ AU}$ (here $M_\odot$ is the solar mass). As this corresponds to a separation of only $\sim 1 \text{ mas}$ on the sky, our images lack the resolution to show such detail directly, and furthermore the infrared flux is so dominated by thermal emission from the warm dust¹¹ that it is unlikely that we have detected the central stars in our maps at all. We can compare our binary parameters with those of the 'episodic' dust producer WR140 which is known to undergo dramatic bouts of dust creation coincident with the passage of a companion star through periastron in a highly elliptical orbit⁹. At periastron, the separation between the stars of WR140 is $\sim 2.5 \text{ AU}$, raising the possibility that the physical conditions favouring copious dust formation fall within a confined range of companion distances for WR + OB binaries.

We may make use of the $1,220 \text{ km s}^{-1}$ wind outflow velocity¹² combined with our proper motion to derive an independent estimate of $2.3 \pm 0.7 \text{ kpc}$ as the distance to WR104, where the dominant error arises from a $\sim 25\%$ uncertainty in outflow velocities found by comparing the results of various line-profile studies¹³ (velocities as high as $1,600 \text{ km s}^{-1}$ have been reported for this star¹⁴). The distance that we determine is somewhat larger than earlier estimates of 1.6 kpc derived from a possible association with Sagittarius OB1 (ref. 15), but the discrepancy is within the estimated errors. Alternatively, if the closer distance is preferred, then our measurements imply an outflow velocity of 845 km s^{-1} for the dust component. Our geometrical solution solves for the projected viewing angle of the observer i , and thus we avoid the usual $\sin(i)$ uncertainty. We note that although isolated dust grains should be momentum-coupled to the flow¹, the outflow velocities in the wake of the passage of the OB stellar companion could be significantly

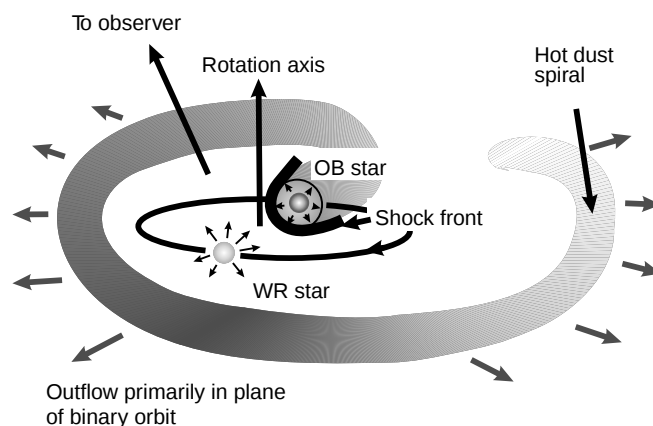


Figure 2 Schematic diagram of the WR104 binary system. The illustration shows the WR star, the OB companion, wind–wind collision front, and the resultant dust outflow plume (not to scale). The spiral shape is a consequence of material being swept radially outwards by the WR wind from a rotating dust nucleation zone associated with the shock front where the stellar winds collide.

perturbed, and thus the behaviour of the plume may not act as a good tracer of the bulk motion of the stellar wind. With additional observations covering an entire orbit, we would be able to greatly refine our estimates of the physical geometry of this system.

It is apparent from Fig. 1 that the outflowing material presents a relatively smooth, spatially confined stream without strong clumping out to a radius of some $\sim 65 \text{ mas}$ ($\sim 150 \text{ AU}$) from the star, by which time the outflow has rotated through $\sim 360^\circ$. We believe that the finding of a single complete turn in the spiral arm is not coincidental, as we detect only dust heated by radiation from the central stars, and therefore lying along a direct line of sight. Material in the second and further coils of the outflow will, of course, be eclipsed by newer material closer in, and will therefore cool rapidly resulting in the relatively sharp cut-off that we see. Although there is some evidence for brightness variations along the arm at a level of a few per cent of the peak, especially apparent in the maps taken at $1.65 \mu\text{m}$, the overall behaviour points to a continuous and smooth dust-creation process, in accord with the classification of WR104 as a 'persistent' dust producer with a constant infrared flux¹⁶. We can again compare this behaviour with that of WR140 whose elliptical orbit results in episodic dust production. The contrasting characteristics of WR104 argue against a high degree of orbital eccentricity, giving some justification to our choice of the archimedean spiral model in this case.

For WR104, our observations confine the infrared excess emission from the dust to lie in a narrow, spatially confined outflow which rotates synchronously with a period of 220 days—a plausible period for a wind-interacting binary system. No spherically symmetric or diffuse component to the dust nebula was detected to within a few per cent of the peak flux. We are therefore able to reject dust-formation models resulting in spherical or disk-shaped outflows, such as clumpy spherical outflows¹⁷ or equatorial density enhancements¹⁸, in favour of the binary wind–wind model.

The viewing angle to the observer of $20 \pm 5^\circ$ is well determined by these measurements. This finding of an almost face-on system contradicts previous attribution of high circumstellar extinction¹⁹ and spectral variability⁹ to an edge-on viewing angle. Some of these observations may be explained as WR104 is thought to lie behind a heavily obscuring cloud¹⁵, with further extinction possibly arising from material created in past mass-loss events of the progenitor star.

As the dust comprises only a very small fraction of the total mass loss, it therefore acts as a visible tracer in the outflow enabling the possibility of dynamical studies of the wind itself. Detailed numerical modelling is needed to determine if the high degree of initial

collimation and subsequent confinement of the dust plume can be explained with simple models of the wind–wind interaction², or whether more detailed three-dimensional calculations are required, such as those of Walder²⁰. Spectral studies of the plume should reveal the thermal and chemical evolution of the dust as it is swept outwards into the interstellar medium, and should also yield information on processes underlying the binary-mediated dust-creation mechanism. With a few dusty WR systems open to study with this method for the detection of binary stars, wider questions of dust formation in this class of objects can now be addressed. □

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Nuclear fusion from explosions of femtosecond laser-heated deuterium clusters

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As a form of matter intermediate between molecules and bulk solids, atomic clusters have been much studied¹. Light-induced processes in clusters can lead to photo-fragmentation^{2,3} and Coulombic fission⁴, producing atom and ion fragments with a few electronvolts (eV) of energy. However, recent studies of the photoionization of atomic

clusters with high intensity ($>10^{16}$ W cm⁻²) femtosecond laser pulses have shown that these interactions can be far more energetic^{5–13}—excitation of large atomic clusters can produce a superheated microplasma that ejects ions with kinetic energies up to 1 MeV (ref. 10). This phenomenon suggests that through irradiation of deuterium clusters, it would be possible to create plasmas with sufficient average ion energy for substantial nuclear fusion. Here we report the observation of nuclear fusion from the explosions of deuterium clusters heated with a compact, high-repetition-rate table-top laser. We achieve an efficiency of about 10^5 fusion neutrons per joule of incident laser energy, which approaches the efficiency of large-scale laser-driven fusion experiments. Our results should facilitate a range of fusion experiments using small-scale lasers, and may ultimately lead to the development of a table-top neutron source, which could potentially find wide application in materials studies.

Fusion neutrons are regularly produced in laser produced plasmas by large-scale laser facilities, usually in the context of inertial confinement fusion (ICF) research. The fusion yield in such experiments can be substantial, for example nearly 5×10^9 DD fusion neutrons are produced in an ICF implosion by the Nova laser at Lawrence Livermore National Laboratory (LLNL) using about 30 kJ of laser energy¹⁴ (although this scheme ultimately scales to much higher efficiency). However, such experiments are limited to large, national laser facilities with low repetition rate lasers (~1 shot per hour). In addition to ICF work, the production of fusion neutrons using various colliding plasma geometries has also been reported^{15,16}: for example, over 10^8 DD fusion neutrons have been produced from shell-confined plasmas using 7 kJ of light from the Gekko XII laser¹⁵. In general, the fusion neutron yield in such experiments is usually $\leq 10^5$ neutrons per joule of laser energy. Petawatt class (10^{15} W) picosecond lasers have recently been shown to produce large amounts of neutrons when focused to very high intensity ($>10^{20}$ W cm⁻²) (ref. 17). Although most of these neutrons are produced by photonuclear processes and exhibit a broad energy spectrum, up to 6×10^4 thermonuclear DD fusion neutrons have been observed from the irradiation of solid, deuterated plastic targets by a 500-J, 5-ps laser. In addition to these results with large scale lasers, small neutron yields have been observed (~140 neutrons per shot) using 100-fs pulses of moderate energy (0.2 J) focused to relativistic intensities ($>10^{18}$ W cm⁻²) on deuterated plastic¹⁸. In this experiment, beam-target neutrons were produced when ions in a preformed plasma were accelerated radially by relativistic channelling of the laser pulse.

The unique nature of the interaction between high-intensity femtosecond laser pulses with atomic clusters can make possible the high ion energies usually achievable only with large-scale, low-repetition rate lasers. When large clusters ($>1,000$ atoms per cluster) are ionized, electrons undergo rapid collisional heating for the short time (<1 ps) before the cluster disassembles in the laser field¹⁹. Through various collective and nonlinear processes, the laser rapidly heats the electrons to a non-equilibrium state (with mean

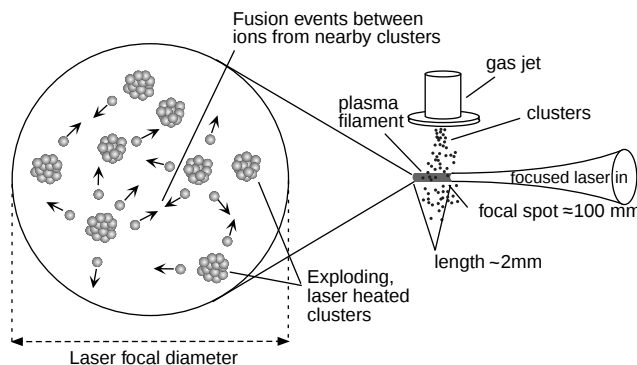


Figure 1 Layout of the deuterium cluster fusion experiment.

energies of many keV) (ref. 8). The escape of these hot electrons from the cluster produces a strong radial electric field which accelerates the cluster ions: the deposited energy is therefore transferred from the light electrons to the more massive ions²⁰. The consequence is that the cluster can very efficiently absorb laser energy (often many tens of keV per atom in the cluster) and this energy is ultimately released in ion kinetic energy when the heated cluster explodes isotropically.

To harness this remarkable release of kinetic energy, we conducted the experiment illustrated in Fig. 1. Here large deuterium clusters are produced in the expansion of a deuterium gas jet plume into vacuum. A high-intensity, ultrafast laser pulse is focused into the gas of deuterium clusters and rapidly heats them. These clusters subsequently explode, ejecting deuterium ions with energies of many keV. This process creates a plasma filament with a diameter that is roughly that of the laser focus ($\sim 200\ \mu\text{m}$) and a length comparable to the extent of the gas-jet plume ($\sim 2\ \text{mm}$). The fast deuterium ions ejected from the exploding clusters can then collide with ions ejected from other clusters in the plasma. If the ion energy is high enough (greater than a few keV), $\text{D} + \text{D}$ nuclear-fusion events can occur with high probability. The well known signature of this process arises from one branch of the fusion reaction, $\text{D} + \text{D} \rightarrow \text{He}^3 + \text{n}$, in which a neutron is released with 2.45 MeV of energy.

We used a table-top laser based on the well known technique of chirped pulse amplification²¹ producing 120 mJ of laser energy in pulses with 35-fs pulse width and a wavelength of 820 nm. This laser fires at a repetition rate of 10 Hz and was focused into the exit of a deuterium gas jet. Because the van der Waals forces between deuterium molecules are weak, the gas jet is cryogenically cooled to -170°C (refs 22, 23): this creates a gas expansion along an adiabat that can produce large clustering in the D_2 jet. From Rayleigh scatter measurements¹⁹, we estimate that the average cluster diameter within the gas jet is about 50 Å. The laser spot size within the gas jet was $\sim 200\ \mu\text{m}$, a size that was found to optimize the fusion neutron yield and which yielded an estimated peak intensity of $2 \times 10^{16}\ \text{W cm}^{-2}$.

To ascertain the efficiency with which the laser energy is coupled into the deuterium clusters, we measured the absorption efficiency of the laser pulse within the deuterium cluster gas jet²⁴. The absorption as a function of gas-jet reservoir backing pressure is shown in Fig. 2a, where the gas has initially been cooled to -170°C (the case in which we observe large D_2 cluster formation): when the deuterium gas-jet pressure rises to $>30\ \text{atm}$, nearly 90% of the laser energy is deposited in the plasma. This contrasts with the energy deposition efficiency of the laser in pure D_2 molecular gas (the case when the gas-jet reservoir is at 20°C): in this case, very little ($<5\%$) of the laser energy is deposited in the plasma, illustrating the importance of the laser/cluster interaction in heating the D_2 plasma²⁴.

To measure the volume and density of the plasma in which this laser energy was deposited, we conducted optical interferometry on the plasma filament²⁵. Using a 35-fs pulse as a probe, we produced an interferometric image of the deuterium plasma within $\sim 20\ \text{ps}$ after the main pulse created the filament, a timescale before the plasma has undergone any significant hydrodynamic motion (one such interferogram is shown in Fig. 2b). Using an Abel inversion, we can find the electron density (and thus the D^+ density) as a function of radius at each point along the filament. This measurement indicates that the average deuterium atom density was $1.5 \times 10^{19}\ \text{cm}^{-3}$. Combining this data with the absorption measurement, we can make a conservative estimate of the average deuterium ion energy by assuming that energy was equally distributed between ions and electrons. (In fact, we expect that more energy is initially transferred to the ions than the electrons in the laser-driven cluster explosion²⁰.) These results indicate that the average deuterium ion energy was at least 2.5 keV. Previous data from exploding clusters indicates that a hot tail of ions exists with energies well above the average energy¹⁰. These multi-keV ions have sufficient energy to drive fusion events.

We detected the production of DD fusion neutrons in our experiment with neutron-sensitive scintillators coupled to photo-multiplier tubes. These detectors were located outside the vacuum interaction chamber and were shielded with 6 mm of lead. We

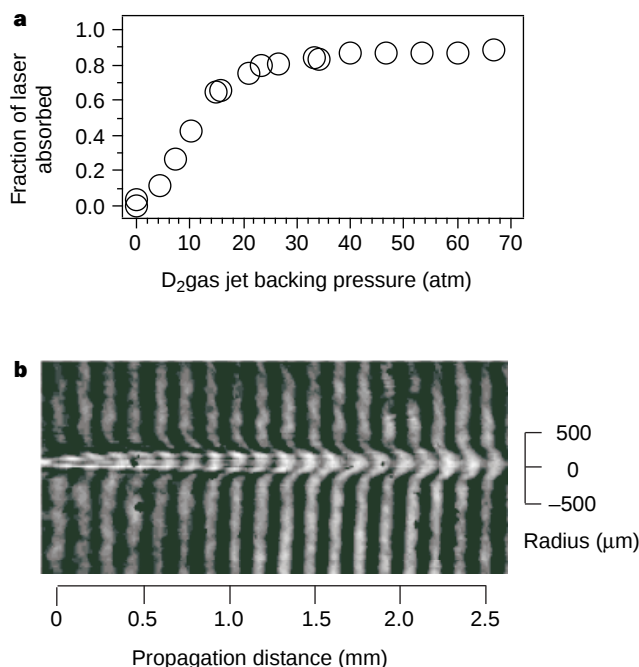


Figure 2 Measured laser absorption and plasma density profile. **a**, Measured absorption fraction of a laser pulse with 120 mJ of energy in the deuterium cluster gas jet as a function of gas-jet reservoir backing pressure. This absorption was found by measuring transmitted and backscattered laser light. (Other scattered light was negligible.) **b**, Interferometric image of the deuterium plasma filament

within $\sim 20\ \text{ps}$ after the main pulse created the filament. The image was recorded by illuminating the plasma with a second probe pulse at a right angle to the propagation of the main pulse. This probe pulse was imaged and sent through a Michelson-type interferometer.

deployed arrays of three detectors at distances of 1, 2.5 and 3.2 m from the plasma. The time of flight (TOF) of particles was recorded. Each neutron detector was operated in a mode such that a neutron was detected every few laser shots, permitting a determination of the flight time of each detected particle with ~ 5 -ns accuracy. With these neutron detectors, we detected the presence of substantial numbers of MeV-energy particles when the gas was backed with cooled deuterium at a pressure of 65 atm and a reservoir temperature of -170°C . The time-of-flight spectrum of particles detected on the neutron detectors is shown for three plasma-detector separations in Fig. 3. For each distance, a distinct peak occurs in the detected TOF spectrum at the position expected for 2.45 MeV neutrons (which have a flight time of 46 ns m^{-1}); this measurement confirms the presence of DD fusion reactions in the cluster plasma. We also confirmed that all detected particles on the neutron detectors disappeared when hydrogen clusters were made in the jet instead of the deuterium clusters (a measurement taken over 50 laser shots, ensuring to better than 1 part in 10^8 that no neutrons are produced when hydrogen replaces deuterium). Furthermore, we found that the fusion neutron production disappeared when deuterium clusters were absent in the D_2 gas jet (when the jet was not cooled). We observed no neutron production in the gas jet with temperatures higher than -120°C , the temperature at which we no longer see large-cluster formation with Rayleigh scattering. These results indicate that the intense illumination of deuterium clusters does give rise to DD fusion.

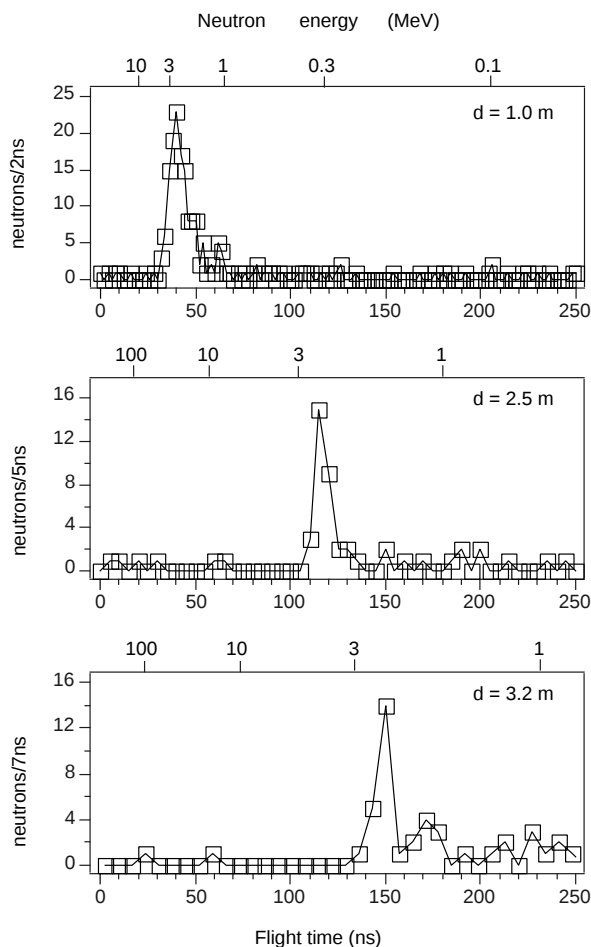


Figure 3 The time-of-flight spectrum of particles detected on the neutron detectors for three plasma-detector separations (1, 2.5 and 3.2 m). Particle flight time is shown on the bottom axes. The energy of a neutron corresponding to the flight time is shown along the top axes. The presence of 2.45-MeV neutrons is clearly visible in all these spectra.

Under the conditions in which the highest yield was attained, there were many multiple hits per shot on the neutron detectors within 2.5 m of the plasma. We were, however, able to estimate the fusion yield by observing the neutron count rate with 3.2 m detection separation, where the detection rate was less than one per shot. Under optimum conditions (found by scanning the laser focus with respect to the gas jet output), we produced $\sim 1 \times 10^4$ neutrons per laser shot. This efficiency ($\sim 10^5$ neutrons per joule of incident laser energy) approaches the fusion neutron production efficiency of large-scale laser-driven fusion experiments.

The expected fusion yield from our experiment can be simply estimated by considering the initial deuteron energies and estimating the disassembly time of the plasma filament. The yield, therefore, is roughly $N \approx (\tau_d/2) \int \langle \sigma v \rangle_T n_D^2 dV$, where $\langle \sigma v \rangle_T$ the velocity-averaged fusion cross-section, and n_D the deuterium density, are integrated over the volume of the plasma; τ_d is the disassembly time of the plasma. As the exact ion-energy distribution is unknown, we assume a maxwellian distribution which, with an average ion energy of 2.5 keV, yields $\langle \sigma v \rangle_T \approx 1 \times 10^{-20} \text{ cm}^3 \text{ s}^{-1}$ (ref. 26). We performed the volume integral using the information from the interferogram. We can estimate the disassembly time as the time required for a ~ 2.5 keV deuteron to exit the plasma of diameter 200 μm : this means the value of τ_d is 200 ps, indicating that the expected fusion neutron yield is $\sim 1.5 \times 10^4$. This magnitude is in reasonable agreement with our measured neutron yield.

It may be possible in the future, to enhance the neutron yield further, perhaps by increasing the ion energies produced from exploding clusters, for example with multiple femtosecond pulses or by using two excitation wavelengths²⁷. It may also be feasible to introduce strong magnetic fields (> 50 T) by using small-gap, pulsed magnets to slow the disassembly time of the hot plasma. Such options could open the way to the production of compact, flexible, high-flux neutron sources for a vast array of uses, with applications in materials science and neutron radiography for example²⁸. □

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Thermodynamic determination of fragility in liquids and a fragile-to-strong liquid transition in water

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If crystallization can be avoided when a liquid is cooled, it will typically form a glass. Near the glass transition temperature the viscosity increases continuously but rapidly with cooling. As the glass forms, the molecular relaxation time increases with an Arrhenius-like (simple activated) form in some liquids, but shows highly non-Arrhenius behaviour in others. The former are said to be ‘strong’ liquids, and the latter ‘fragile’^{1,2}. Here we show that the fragility of a liquid can be determined from purely thermodynamic data (as opposed to measurements of kinetics) near and below the melting point. We find that for most liquids the fragilities estimated this way are consistent with those obtained by previous methods and by a new method (ref. 3 and K.I., C.A.A. and C.T.M., unpublished data) at temperatures near the glass transition. But water is an exception. The thermodynamic method indicates that near its melting point it is the most fragile of all liquids studied, whereas the kinetic approach indicates that near the glass transition it is the least fragile. We propose that this discrepancy can be explained by a fragile-to-strong transition in supercooled water near 228 K, corresponding to a change in the liquid’s structure at this point.

One of the intriguing (and still unresolved) questions about supercooled liquids was posed in 1948 by Kauzmann⁴, who showed that many liquids lose entropy on supercooling so much faster than the corresponding crystalline phase that an ‘entropy crisis’, where $S_{\text{liq}} < S_{\text{cryst}}$, should occur at temperatures as high as $0.67T_m$ (here S_{liq} and S_{cryst} are the entropies in the liquid and the crystal, respectively, and T_m is the melting point). This situation is averted only by the occurrence of the glass transition. The entropy crisis is most pressing for the fragile liquids. We reproduce in Fig. 1 Kauzmann’s plot of the entropy difference ΔS between the liquid and the crystal normalized to the entropy difference at the melting point, $\Delta S/\Delta S_m$, versus normalized temperature T/T_m . Newer data have been added for the very fragile salt hydrate, $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ (refs 5–7), for the less fragile molecular liquid bromopentane^{8,9}, and for the rather strong covalent liquid As_2Se_3 (refs 2, 10 and S. S. Chang, personal communication). The temperature where $\Delta S/\Delta S_m \rightarrow 0$ by extrapolation, known as the Kauzmann temperature T_K , is the effective ground-state temperature for each liquid. Consistent

with the idea of fragility as a rate-of-structural-change metric, the liquids that on cooling approach their T_K most rapidly are seen also to be the liquids in the original ‘fragility plot’^{1,2} whose viscosities, plotted against T_g/T , approach infinity most rapidly (here T_g is the glass transition temperature).

The correspondence of fragilities based, alternatively, on kinetic properties with those based on purely thermodynamic properties, is consistent with the notion advanced by Gibbs¹¹ and Goldstein¹² that the dynamics of liquids largely reflect the underlying thermodynamics of the liquid state. This idea has recently been strongly supported by the ‘inherent structure’ or ‘landscape’ analysis of Sastry *et al.*¹³, for a model system studied by computer simulation. Figure 1 has the advantage that it can include liquids which, like water, do not have good glass-forming ability owing to fast crystallization kinetics. Data for liquid water at temperatures down to -35°C (ref. 14) are included in Fig. 1. By the standard of Fig. 1, water is seen to be the most fragile liquid of all, notwithstanding a subtle effect which makes easily crystallizing liquids appear less fragile than they would on the normal (T_g -scaled) ‘fragility plot’.

The nature of the data in Fig. 1 invites fragility for glass-formers to be quantified by the ratio T_g/T_K , by analogy with the metric $F = T_0/T_g$ (where T_0 is the relaxation time divergence temperature of the Vogel–Fulcher–Tammann equation) suggested by some authors^{15,16} for relaxation-based (kinetic) fragilities. An ambiguity which is common to these two fragility metrics arises from the need for extrapolations to obtain T_K and T_0 . The ambiguity can be removed in each case by defining $F_{1/2}$ fragilities. In the case based on relaxation time, $F_{1/2}$ has been defined⁹ using the temperature $T_{1/2}$ at which the relaxation time on a log scale is halfway between its

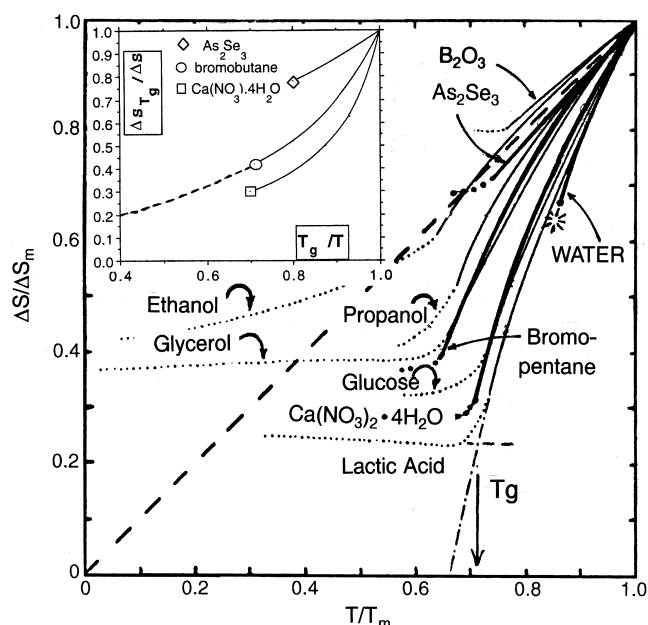


Figure 1 Use of the Kauzmann plot to define thermodynamic fragility for glass-forming liquids. The plot shows how the immediacy of the entropy crisis for liquids cooled below their melting temperatures follows the same order as the fragilities of these liquids as determined from the familiar T_g -scaled Arrhenius plot of transport data. Data for supercooled water on the same plot show the most fragile behaviour of all, despite a distortion towards lower fragility caused by the high melting-to-glass temperature ratio for water. The temperature of homogeneous nucleation for $\sim 2\text{-}\mu\text{m}$ emulsion droplets is marked by a star. Inset, alternative representation of data in main panel, which emphasizes the close relation of thermodynamic fragility to the more familiar (kinetic) fragility plot. Data for bromobutane were extended beyond the melting point in ref. 8. In this alternative plot, non-glassformers are excluded unless the scaling temperature T_g is obtained by estimation.

value at T_g ($\sim 10^2$ s) and the value at infinite temperature (phonon timescale) ($\sim 10^{-14}$ s). The definition, which gives fragility values between 0 and 1, is $F_{1/2} = 2(T_g/T_{1/2}) - 1$. A thermodynamic fragility, $F_{1/2,cal}$ (with values between zero and unity) can be likewise defined as $F_{1/2,cal} = T_{1/2}/T_m$, where $T_{1/2}/T_m$ is the T/T_m value where half the fusion entropy ΔS_m has been lost on cooling. However, “strong” glass-formers like B_2O_3 and As_2Se_3 can then only be included by extrapolation (see Fig. 1). An alternative and less ambiguous definition of thermodynamic fragility could be the fraction of the fusion entropy lost by $T/T_m = 0.8$, which can be determined experimentally for all the liquids in Fig. 1 except water. Water, by extrapolation, would have the highest fragility of the liquids shown in Fig. 1 (in fact it would be off-scale). Likewise, if we adopted the T_g implied by power-law fits of the various water relaxation data in the temperature range of the Fig. 1 entropy data, a uniquely high kinetic fragility, $F_{1/2}$, would be obtained for water. Even using the T_g value of 136 K obtained from calorimetric measurements on amorphous water—but which we will argue is inappropriate for the scaling of water data above 236 K—the fragility of water is high, though not uniquely so.

Before discussing the unusual properties of water in detail, we point out that, despite appearances, the data of Fig. 1 are effectively temperature-scaled by T_g , as in the common kinetic fragility plot. This is because, except for water, the substances in Fig. 1 obey the so-called “2/3 rule” of glass-formers, which says that T_g is approximately $(2/3)T_m$. Indeed, if the data of Fig. 1 are replotted in the form $\Delta S_T/\Delta S$ versus T_g/T , where ΔS_T is the value of ΔS at T_g , the resemblance to the standard fragility plot is striking, as shown in Fig. 1 inset. The establishment of a thermodynamic fragility scale for liquids, with its implications for the liquid density of states (vibrational and configurational)¹⁷, is an important objective of this work, but our main aim is to consider the unusual behaviour of water.

A glassy state of water would be expected to result from any cooling procedure that bypasses the crystallization at $0.85T_m$ (the star-burst symbol in Fig. 1). Assuming that such water followed the trend in Fig. 1 (increasing T_g/T_m with increasing steepness of the $\Delta S/\Delta S_m$ versus T/T_m plot), one would estimate for normal cooling rates near the glass transition that T_g for water would lie in the range roughly 200–220 K, depending on the chosen extrapolation of the (rapidly changing) liquid heat-capacity data used to calculate $\Delta S/\Delta S_m$. However, what is known from an abundance of binary solution glass-transition data^{18,19}, and from less definitive measurements on hyperquenched water itself^{20,21}, is that the glass transition of water is located at ~ 136 K. Furthermore, as we will show, the liquid which vitrifies at this temperature is a very strong liquid, notwithstanding binary solution extrapolations which predict the opposite¹⁹. Something strange happens to water on cooling between 236 and 150 K, or on addition of solutes at concentrations between zero and 20 mol%.

To demonstrate convincingly that water which has been obtained in the glassy state by very rapid cooling gives a kinetically “strong” liquid above its T_g , we need to obtain data on the temperature dependence of its structural relaxation time in this domain. This is usually obtained by some viscosity measurement or dielectric relaxation-time determination, but satisfactory data are not yet available for pure water. However, a simple alternative is available, as we will show below.

The glass transition is a relaxation phenomenon, and the decrease on cooling of the heat capacity from its equilibrium liquid value to the glassy value (and vice versa on reheating) occurs over a range of temperature; the range is determined primarily by how quickly the relaxation time changes with temperature. The transition extends over the temperature range needed to change the relaxation time by some 1.5–2.5 orders of magnitude^{3,22}, depending on the glass and on how the width of the glass transition is defined. A correlation of the glass transition width $\Delta T_g (= T'_g - T_g)$ from scanning calorimetry (Fig. 2, left inset) with the activation energy for viscosity η —which

is tantamount to a correlation of fragility with reduced glass transition width $\Delta T_g/T_g$ —has been demonstrated for inorganic glasses³. We have extended this correlation to molecular glasses using precise dielectric relaxation data instead of viscosities to define the $F_{1/2}$ fragilities. A combination of these results is shown in Fig. 2. The line shows the dependence of $\Delta T_g/T_g$ on $F_{1/2}$ predicted using the Vogel–Fulcher–Tammann equation for the temperature dependence of the relaxation time τ and the viscosity η ; this prediction uses the assumptions that the glass transition range ΔT_g (defined above) corresponds to a change of 2.3 decades in τ and η , and that τ and η at the onset T_g lie 16 decades above the microscopic (phonon) time τ_0 and the high-temperature limit of η , as all the data indicate^{1,2}. Some details are given in Fig. 2 legend.

What can this tell us about water near its glass transition, generally identified as the low-density amorphous form of ice? We are particularly interested in the case of the material obtained by aerosol quenching of the liquid state, called HQG (hyper-quenched

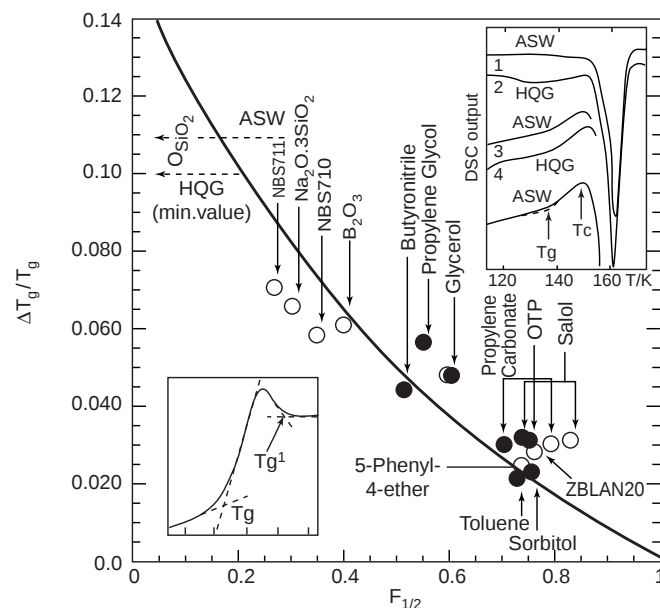


Figure 2 Correlation between fragility metrics $\Delta T_g/T_g$ and $F_{1/2}$. Shown is an extended correlation of the reduced glass transition width $\Delta T_g/T_g (= (T'_g - T_g)/T_g$, see left inset) with the $F_{1/2}$ fragility (defined in text) obtained from precise dielectric relaxation data for molecular liquids (for example, ref. 9) (filled circles) and viscosity data for inorganic compounds³ and some molecular liquids (open circles). NBS 711 and 710 are (former NBS) standard silica-based glasses, ZBLAN20 is a complex zirconium fluoride based glass, and OTP is *ortho*-terphenyl. Dashed arrows to the vertical axis show the $\Delta T_g/T_g$ of hyperquenched glassy water (HQG) and vapour-deposited glassy water (ASW), the minimum values of which were determined as shown in the right inset, following ref. 21 (T_c is the temperature at which the glass transition is cut short by crystallization). Comparison with the value for SiO_2 , or reference to the correlation line for all systems, identifies vitreous water as a very ‘strong’ liquid. The $\Delta T_g/T_g$ values obtained from the curves in the right inset are minimum values because (1) the annealing treatment used by Hallbrucker *et al.*²¹ to sharpen the glass transition usually reduces its width, and (2) crystallization may have occurred before the transition was complete. The correlation line in is a relation between $\Delta T_g/T_g$ and $F_{1/2}$ calculated using the methodology of ref. 3, which assumes that the temperature dependence of τ or η can be reasonably approximated by the Vogel–Tammann–Fulcher equation. In addition, it was assumed in the calculation that τ and η change by 16 orders of magnitude between T_g and their high temperature limits and by 2.3 orders of magnitude between T_g and T'_g , from which we derive $\Delta T_g/T_g = 0.151(1 - F_{1/2})(1 + F_{1/2})$. Because these assumptions are valid only to varying degrees of approximation for different liquids, the scatter of the data points about the correlation line is expected.

glass)^{20,21}, because, in this path to the glass, the sample is continuously liquid. However the findings are much the same for other amorphous waters, such as vapour-deposited amorphous solid water (ASW), as seen in Fig. 2, right inset. The differential scanning calorimetry (DSC) data for HQG and ASW, after an annealing treatment to remove the excess enthalpy frozen-in during the hyperquench (followed by further annealing below T_g to enhance the transition strength to a detectable level²¹), are shown in Fig. 2, right inset. The reduced-width $\Delta T_g/T_g$ found is 0.10 and is remarkably large—as large as that of the archetypal strong liquid, SiO_2 . This reduced-width value is marked by a horizontal dashed arrow in Fig. 2. From comparison with the $\Delta T_g/T_g$ for silica or from the intersection with the $\Delta T_g/T_g$ versus $F_{1/2}$ correlation line, the $F_{1/2}$ value for low-temperature liquid water is seen to be very small. Indeed, taking the thermal treatment into account (see Fig. 2 legend), this water must be the strongest liquid yet identified. In this sense, it is quite distinct as a liquid from the water observed above 230 K (see Fig. 1).

Whereas the reduced- T_g -width approach that we have used here is the most direct way at present available of identifying HQG water as a 'strong' liquid, there are several ways of supporting this conclusion, though they cannot be detailed here. One is from the enthalpy relaxation activation energy of $\sim 50 \text{ kJ mol}^{-1}$ cited by Hofer *et al.*²³, which translates via simple equations to $F_{1/2} \approx 0.05$. Another invokes the thermodynamic fragility introduced earlier by comparing the tiny value ($1.6 \text{ J mol}^{-1} \text{ K}^{-1}$; ref. 21) of the excess heat capacity ($C_p(\text{liq.}) - C_p(\text{glass})$) measured across the glass transition (Fig. 2 insert) with the very large value for supercooled water above 236 K (ref. 14).

Thus the difference in character of water above 236 K and below 150 K, that is, in the observable ranges, is real and extreme. So what is implied about the events occurring in water during continuous, if very fast, cooling from normal temperatures?

To answer this we turn to the second of the two possibilities discussed with equal weight by Speedy and Angell in their original analysis of the apparently divergent properties of supercooled water²⁴. As an alternative to the much-discussed, and now disputed, stability limit interpretation²⁴ (which corresponds to the system reaching the 'edge' of its free-energy surface, $G(P,T)$) Speedy and Angell proposed that running across this surface might be a ridge associated with an end-point in the open hydrogen-bonded network formation process. This process was seen as "geometrically cooperative"²⁴, which would give rise to a region of rapid entropy change. Expected would be a peak in the heat capacity, followed by a drop to very small excess heat capacity, due to the exhaustion of the network degrees of freedom—as envisaged in ref. 14. The existence of an extremum in compressibility has been shown²⁵ to be a thermodynamic consequence of the existence of a density maximum, and must be observed in water somewhat below 4°C unless pre-empted by some other phenomenon. Possible pre-emptives would be a first-order phase transition, as seen in liquid silicon^{26,27}, or the glass transition, as observed in liquid SiO_2 (ref. 28), below their respective ambient-pressure density maxima.

Theory²⁹ shows C_p extrema must also occur, but not necessarily at the same temperature as that of the compressibility maximum. However, an analysis³⁰ of available thermodynamic data constrains the C_p maximum to lie fairly close to 228 K, if the changes are to be continuous. Below such a maximum, the C_p of water must fall to the low value observed, and then the Adam–Gibbs equation³¹—which has so far proved quite successful for liquids near their T_g (refs 9, 32)—would require the liquid to be 'strong' in character.

The Adam–Gibbs theory connects the temperature dependence of the relaxation time or of a related transport property to the variation with temperature of the configurational entropy S_c via the relation

$$\tau = \tau_0 \exp(C/TS_c) \quad (1)$$

where τ_0 and C are constants. It has been shown³³ to describe well the anomalously large and non-Arrhenius temperature dependence of the diffusion coefficient of water above 243 K. Near T_g , with almost no heat capacity in excess of the vibrational component, equation (1) would predict very different behaviour. S_c would be almost constant, and an Arrhenius temperature dependence of transport properties corresponding to a 'strong' liquid, as indicated in the present work, would be found for any relaxation or transport process coupled to the entropy fluctuations responsible for the C_p behaviour. Thus the fragile-to-strong liquid transition on cooling is intimately connected to the presence of a thermodynamic event in liquid water. We note that near a critical point in a two-component liquid, self-diffusivity does not follow viscosity or mutual diffusion³⁴, so it is possible that the diffusivity^{35,36} of water could, as a decoupled background mode, be an exception to these expectations.

Inverting the analysis of ref. 26, Roberts *et al.*³⁷ recognize that the thermodynamic event in water referred to above would require a density extremum at a higher temperature. Hence this event could account for the existence of the density maximum of water at 4°C . $\square$

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Control of crystal nucleation by patterned self-assembled monolayers

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An important requirement in the fabrication of advanced inorganic materials, such as ceramics and semiconductors, is control over crystallization^{1–4}. In principle, the synthetic growth of crystals can be guided by molecular recognition at interfaces^{5–16}. But it remains a practical challenge to control simultaneously the density and pattern of nucleation events, and the sizes and orientations of the growing crystals. Here we report a route to crystal formation, using micropatterned self-assembled monolayers^{17,18}, which affords control over all these parameters. We begin with a metal substrate patterned with a self-assembled monolayer having areas of different nucleating activity—in this case, an array of acid-terminated regions separated by methyl-terminated regions. By immersing the patterned substrates in a calcium chloride solution and exposing them to carbon dioxide, we achieve ordered crystallization of calcite in the polar regions, where the rate of nucleation is fastest; crystallization can be completely suppressed elsewhere by a suitable choice of array spacing, which ensures that the solution is undersaturated in the methyl-terminated regions. The nucleation density (the number of crystals formed per active site) may be controlled by varying the area and distribution of the polar regions, and we can manipulate the crystallographic orientation by using different functional groups and substrates.

We patterned self-assembled monolayers (SAMs) on the metal substrates by microcontact printing with an elastomeric stamp^{18,19} that had a relief structure consisting of a square array of raised circles with diameter d and periodicity p (Fig. 1a): as “inks”, we used 10 mM solutions of $\text{HS}(\text{CH}_2)_n\text{X}$ ($\text{X} = \text{CO}_2\text{H}$, SO_3H , OH) in ethanol. The surface was then washed with a 10 mM solution of $\text{HS}(\text{CH}_2)_{15}\text{CH}_3$ in ethanol to passivate the areas that had not contacted the stamp. We focus on the crystallization of calcite (CaCO_3). The general principles of calcite formation have been extensively studied, due to the importance of this mineral in nature as a structural material^{20,21}. The patterned substrates were supported upside-down in a calcium chloride solution to ensure that only particles grown on the SAM would be bound to the surface, and placed in a closed desiccator with vials of solid ammonium carbonate in the bottom⁶ (Fig. 1b).

The surfaces decorated with crystals were examined using a scanning electron microscope (SEM) operating at 15 keV. The crystallographic orientations of the crystals relative to the surface were determined using X-ray diffraction and morphological

analysis²⁰. Figure 1c shows a low-magnification micrograph of the pattern of calcite crystals formed on a sample SAM—printed 35- μm circles of $\text{HS}(\text{CH}_2)_{15}\text{CO}_2\text{H}$ in a background of $\text{HS}(\text{CH}_2)_{15}\text{CH}_3$ —supported on Ag ($[\text{Ca}^{2+}] = 25 \text{ mM}$, crystallization time $t = 30 \text{ min}$). Crystallization is restricted to well defined, CO_2^- -terminated regions, and does not occur on the CH_3 -terminated area of patterned surfaces, although the solution was supersaturated in CaCO_3 and crystal formation occurred on both CO_2^- - and CH_3 -terminated SAMs when they were not patterned (Fig. 1c, insets).

By adjusting the pattern, density and sizes of features in the stamp, the concentration of the crystallizing solution, and the

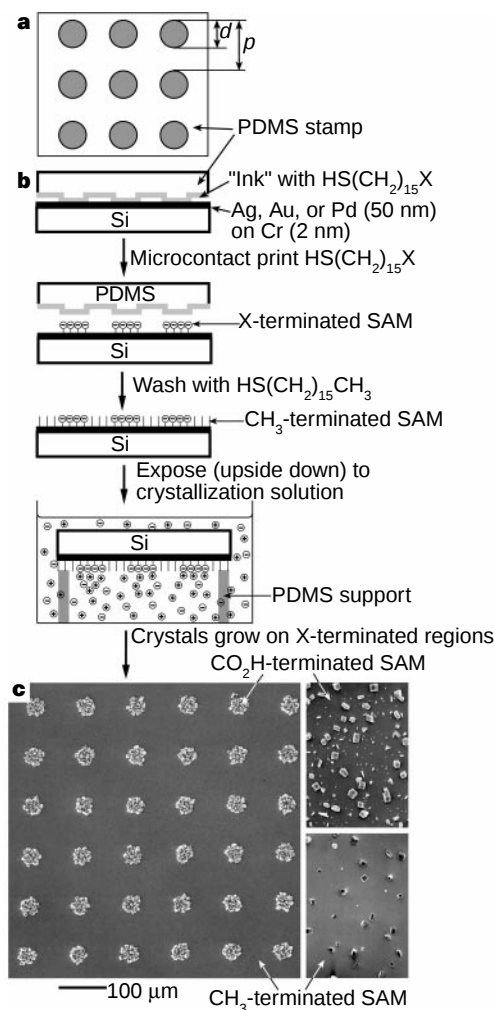


Figure 1 Experimental design of crystallization on patterned SAMs. **a**, Relief structure of the patterned PDMS stamps used for microcontact printing. **b**, Schematic presentation of the experimental steps. Dimensions are not to scale. **c**, Scanning electron micrograph (SEM) of the sample patterned surface—printed circles of $\text{HS}(\text{CH}_2)_{15}\text{CO}_2\text{H}$ in a background of $\text{HS}(\text{CH}_2)_{15}\text{CH}_3$ supported on Ag(111)—overgrown with calcite crystals. For these experimental conditions— $[\text{Ca}^{2+}] = 25 \text{ mM}$, $\text{pH} \sim 7$, crystallization time $t = 30 \text{ min}$ —the nucleation is highly specific to the acid-terminated regions, and the crystals are remarkably uniform in size and nucleation density. The insets illustrate the wide distribution of sizes of crystals formed on the non-patterned SAMs presenting the same terminal groups to a solution with the same value of $[\text{Ca}^{2+}]$. To prepare substrates, silicon wafers (test grade, n or p type, Silicon Sense, Nashua, NH) were coated with 2.5 nm of Cr, to promote adhesion, and then with metal (Ag, Au, Pd; typically $\sim 50 \text{ nm}$) using an electron beam evaporator (base pressure 10^{-7} torr). The stamps were prepared by casting and curing poly(dimethylsiloxane) (PDMS) against rigid masters bearing a photoresist pattern formed using conventional lithographic techniques^{18,19}. Calcite crystals formed on diffusion of carbon dioxide and ammonia vapours into the CaCl_2 solution.

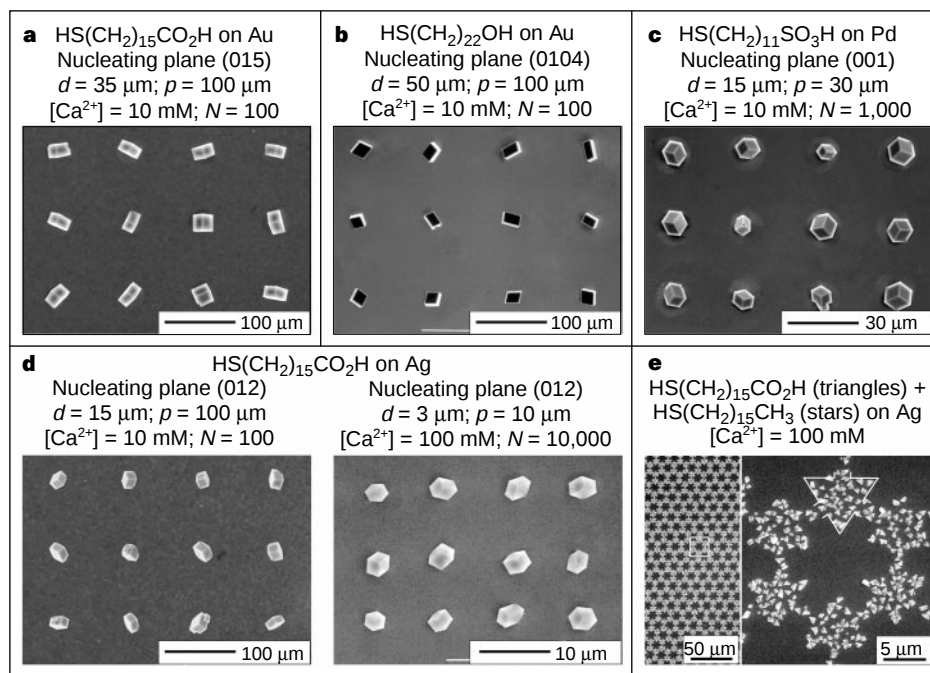


Figure 2 Ordered two-dimensional arrays of single calcite crystals. The densities of nucleation, uniform sizes and crystallographic orientation are controlled by the micropatterned SAMs consisting of regions of $\text{HS}(\text{CH}_2)_n\text{X}$ and $\text{HS}(\text{CH}_2)_{15}\text{CH}_3$. The density and sizes of features in the stamp and the concentration of the crystallizing solution were chosen to ensure the formation of one crystal per printed site. **a**, Arrays of crystals with the density of nucleation $N \approx 100 \text{ crystals mm}^{-2}$ grew selectively from the (015) plane on SAMs of $\text{HS}(\text{CH}_2)_{15}\text{CO}_2\text{H}$ supported on Au(111). **b**, Arrays of crystals with the density of nucleation $N \approx 100 \text{ crystals mm}^{-2}$ grew selectively from the (104) plane on SAMs of $\text{HS}(\text{CH}_2)_{22}\text{OH}$ supported on Au(111). **c**, Arrays of crystals with the density of

nucleation $N \approx 1,000 \text{ crystals mm}^{-2}$ grew selectively from the (001) plane on SAMs of $\text{HS}(\text{CH}_2)_{11}\text{SO}_3\text{H}$ supported on Pd. **d**, Arrays of crystals nucleated selectively from the (012) plane on SAMs of $\text{HS}(\text{CH}_2)_{15}\text{CO}_2\text{H}$ supported on Ag(111) with various densities of nucleation: $N \approx 100 \text{ crystals mm}^{-2}$ (left) and $N \approx 10,000 \text{ crystals mm}^{-2}$ (right). **e**, An example of the fabrication of another complex crystalline pattern: a continuous, polycrystalline structure formed on SAMs consisting of a hexagonal array of 'stars' of $\text{HS}(\text{CH}_2)_{15}\text{CH}_3$ ($d = 12 \mu\text{m}$, $p = 15 \mu\text{m}$) in a field of $\text{HS}(\text{CH}_2)_{15}\text{CO}_2\text{H}$ on Ag(111). The low-magnification SEM (left) illustrates the high fidelity of the procedure, and the high-magnification fragment (right) shows the formation of uniform crystals of sub-micrometre sizes.

functionality of the surface of the SAM, we could control the most important characteristics of the crystallization process: that is, the location and density (N) of nucleating regions on the surface, the number (n) of crystals that nucleated within each region, and the crystallographic orientation of these crystals. Figure 2a–d shows several examples of ordered arrays of single calcite crystals ($n = 1$) with controllable densities of nucleation ($N \approx 100, 1,000$ and $10,000 \text{ crystals mm}^{-2}$) and crystallographic orientation (crystals nucleated specifically from the (015), (104), (001) and (012) planes on SAMs of Au/ $\text{HS}(\text{CH}_2)_{15}\text{CO}_2\text{H}$, Au/ $\text{HS}(\text{CH}_2)_{22}\text{OH}$, Pd/ $\text{HS}(\text{CH}_2)_{11}\text{SO}_3\text{H}$ and Ag/ $\text{HS}(\text{CH}_2)_{15}\text{CO}_2\text{H}$, respectively).

Control of orientation is based on our observation that SAMs terminated in different functional groups, as well as SAMs of a single thiol supported on different metal substrates, induce calcite nucleation from specific crystallographic planes and cause growth to occur in different crystallographic directions. The orientation of crystals is highly uniform for each SAM (the percentage of oriented crystals, estimated by morphological analysis of five random areas bearing ~ 40 crystals each, was 97–98% with standard deviation of 2–3%), an observation implying that the atomic-level interfacial structure is controlling the nucleating plane of the crystals. The selectivity of the oriented growth of calcite induced by the X-terminated SAMs is higher than that induced by Langmuir monolayers or polymer surfaces terminated in the same functional groups^{6,7,11,12,16}, due, we presume, to the more ordered structure of SAMs. SAMs terminated in different functional groups and/or supported on different substrates have different structural parameters¹⁷. The correlation between the structures of SAMs and crystal planes they nucleate suggests that face-selective nucleation stems from a match between the pattern and orientation of ions adsorbed on the organic surface and those in the nucleating crystal plane^{6,9,10}. The method described

here makes it possible, to our knowledge for the first time, to combine area-selective nucleation with highly specific growth of crystals in a single crystallographic direction.

The ability to form one crystal per active site ($n = 1$) is based on our observation that at constant concentration, the number of crystals, n , within each active nucleation region depended linearly upon its area (Fig. 3). This relationship makes it possible to determine the value of n by choosing the size, d , of the raised features in the stamp (Fig. 1a), and therefore provides precise control over the nucleation density.

A comparison between the arrays of crystals grown on patterned surfaces (Fig. 2a–d) and arrays grown on homogeneous, non-patterned SAMs (Fig. 1c, insets) highlights another advantage of this method—formation of crystals with similar sizes that reflect nucleation in different locations at similar times. We infer, therefore, that patterning surfaces with SAMs in regular arrays makes it possible to achieve control over many of the crucial aspects of nucleation and crystal growth. To our knowledge, Fig. 2 shows the first examples of periodic arrays of discrete crystals with controlled densities and location of nucleation, and with uniform sizes and crystallographic orientations. These arrays formed reproducibly in more than 10–20 repetitions.

This method is not limited to the nucleation of crystals on isolated regions. We are able to grow calcite crystals on SAMs having a range of patterns with sizes of features from submillimetre to submicrometre. Figure 2e shows an example of a complex pattern of calcite crystals grown on a surface having a pattern of interconnected triangles of carboxylic-acid-terminated SAM in a field of methyl-terminated SAM; the crystals follow the geometry of the underlying active nucleating regions with good fidelity. An earlier attempt to perform patterned crystallization on electron-beam-

damaged SAMs¹³ resulted in edge resolution of 2 μm . Our method generates patterns of crystals with edge resolution of a few hundred nanometres.

We suggest that high-resolution crystallization on the X-terminated regions—and the absence of nucleation on the CH_3 -terminated regions—of patterned SAMs reflects the influence of mass transport. The rate of nucleation on SAMs terminated in polar groups is faster than on methyl-terminated SAMs. As soon as crystal growth begins in a polar region, mass transport to the growing crystals depletes calcium and carbonate ions over the local methyl-terminated region to the point of undersaturation. In surface growth models, the regime in which growth occurs on the surface with predeposited islands is usually referred to as submonolayer, or island, epitaxy²². The model that describes the growth behaviour in such systems—the DDA (deposition, diffusion and aggregation) model—predicts a characteristic length scale for a diffusion-limited, island-specific epitaxy: $l_d \approx (D/F)^{1/4}$, where l_d (cm) defines the size of the region where deposition does not occur, D ($\text{cm}^2 \text{s}^{-1}$) is a diffusion coefficient and F ($\text{cm}^{-2} \text{s}^{-1}$) is a flux of particles (ions, atoms or molecules). In our system, l_d corresponds to the distance from the active nucleating region at which the local concentration of the solution (depleted by the mass transport to the growing crystals) approaches the saturation level for the nucleation of calcite on the slowly nucleating region of the SAM (Fig. 4a). The inhibition of nucleation would, therefore, appear only within a distance, l_d , from the edge of the active region. To test this conclusion, we performed crystallization on a methyl-terminated surface with one isolated region terminated in carboxylic acids. The experimental conditions— $[\text{Ca}^{2+}] = 25 \text{ mM}$, $d = 35 \mu\text{m}$, crystallization time $t = 30 \text{ min}$ —were chosen to correspond to Fig. 1c. Solving the diffusion equation, $\partial c/\partial t = D \partial^2 c/\partial x^2$

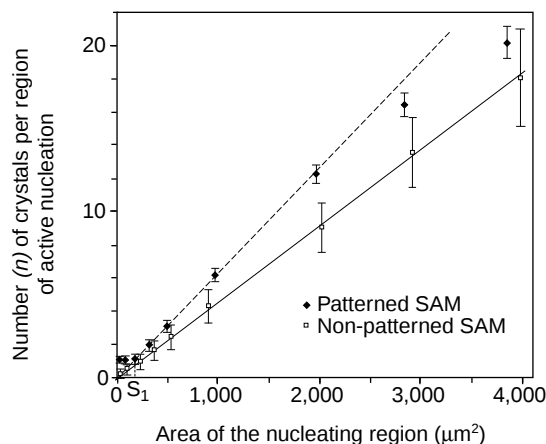


Figure 3 Average number of crystals nucleated per active region as a function of area, for patterned and non-patterned SAMs. The difference in standard deviation reflects the higher uniformity of the nucleation density on patterned SAMs than on non-patterned SAMs. For a large range of sizes of active regions of patterned SAMs ($d = 15\text{--}50 \mu\text{m}$), the average number of crystals nucleated per active region, n , is proportional to the area of the nucleating region (dashed line). As d increases, the nucleation densities decrease and approach the corresponding values for non-patterned SAMs (solid line). For small nucleating regions ($S \leq S_1$), the number of crystals nucleated per active region is constant ($n = 1$) irrespective of the area; this observation suggests that the size of the printed features is much larger than the critical size of the nucleation site for calcite. Calcite crystals were grown on nine patterned surfaces consisting of printed circles of $\text{HS}(\text{CH}_2)_{15}\text{CO}_2\text{H}$ in a background of $\text{HS}(\text{CH}_2)_{15}\text{CH}_3$ supported on Ag(111) (diameters of circles, d , were 5, 10, 15, 20, 25, 35, 50, 60 and $70 \mu\text{m}$). For each surface, one 7×7 -array of CO_2H -terminated circles was used for statistics (49 points) to determine the average number of crystals per nucleating spot and the standard deviation. We compared these data to the statistical results of the growth of calcite on non-patterned SAMs of $\text{HS}(\text{CH}_2)_{15}\text{CO}_2\text{H}$ supported on Ag(111).

(where c is the concentration and x is the position), for these specific parameters and assuming that the concentration of the saturated solution ($c_{\text{sat}} \approx 2\text{--}2.5 \text{ mM}$), we estimated the size of the depletion region as $l_d = 80\text{--}100 \mu\text{m}$. Indeed, in Fig. 4b crystallization of calcite is observed in the predicted pattern, with no detectable nucleation within $\sim 80 \mu\text{m}$ of the active region. Outside this area, nucleation occurs in a manner similar to that on non-patterned methyl-terminated surfaces (Fig. 1c, inset). For distances between the features, $p < 2l_d$, the solution over the slowly nucleating regions is effectively undersaturated (Fig. 4c), and there is no nucleation in these areas (as shown in Fig. 1c, where $p = 100 \mu\text{m} < 2l_d = 160 \mu\text{m}$).

Diffusion-controlled growth of calcite in nature is believed to be controlled locally by specialized macromolecules²¹. The power of our approach to crystallization is, therefore, its ability simultaneously

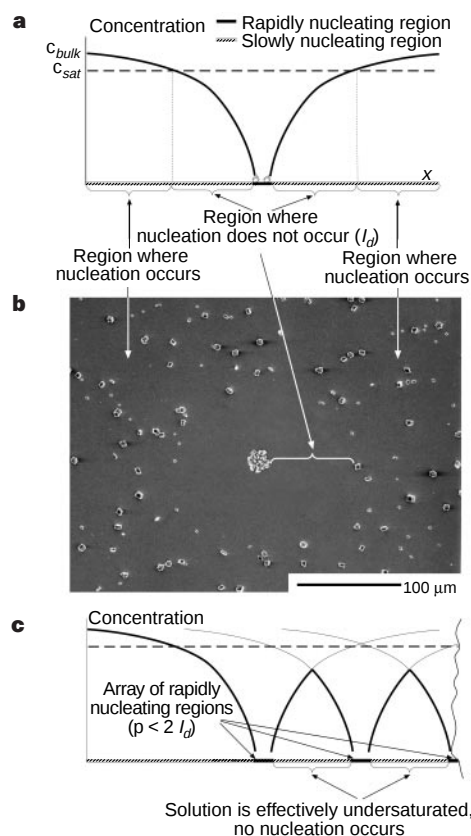


Figure 4 The effects of diffusion on nucleation. **a**, Calculated profiles of concentration of the crystallizing solution ($c(x)$) in the vicinity of the isolated, rapidly nucleating region, assuming mass transport to the preformed, growing crystal (crystals). The profiles were derived using the diffusion equation: $\partial c/\partial t = D \partial^2 c/\partial x^2$, and assuming zero concentration of solution at the surface of the crystallizing region (that is, assuming that all the ions that reach the surface of the crystal stick irreversibly). The dashed line corresponds to the concentration of the saturated solution, c_{sat} , below which nucleation on the slowly nucleating surface does not occur. In the region l_d where $c(x) < c_{\text{sat}}$, nucleation is, therefore, suppressed. Nucleation is allowed for distances from the rapidly nucleating region, $x > l_d$, where $c > c_{\text{sat}}$. For the nucleation on the methyl-terminated surfaces, c_{sat} is $\sim 2.5 \text{ mM}$. **b**, SEM image of the pattern of calcite crystals grown on a methyl-terminated surface with one isolated carboxylate-terminated region, showing the depletion distance, $l_d \approx 80 \mu\text{m}$, in agreement with the value ($\sim 80\text{--}100 \mu\text{m}$) calculated assuming $c_{\text{bulk}} = 25 \text{ mM}$, $c_{\text{sat}} = 2\text{--}2.5 \text{ mM}$ and crystallization time $t = 30 \text{ min}$. **c**, Calculated profiles of the concentration of the crystallizing solution in the vicinity of an array of rapidly nucleating regions with periodicity $p < 2l_d$. The effective concentration (bold lines) over the entire slowly nucleating region is then below c_{sat} . Crystallization will only take place on the rapidly nucleating regions, as shown in Fig. 1c.

to control the microenvironment of the nucleation site and to manipulate near-surface gradients of concentrations of the crystallizing ions by patterning SAMs into rapidly and slowly nucleating regions.

The technique we report here gives us the ability to fabricate a large number of indistinguishable active nucleation regions, and to nucleate one crystal in each region. This should enable the study of fundamental aspects of the crystallization process by providing access to statistically significant numbers of nucleation events in highly controlled microenvironments. □

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Complete asymmetric induction of supramolecular chirality in a hydrogen-bonded assembly

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Chirality at the supramolecular level involves the non-symmetric arrangement of molecular components in a non-covalent assembly^{1,2}. Supramolecular chirality is abundant in biology, for example in the DNA double helix³, the triple helix of collagen⁴ and the α -helical coiled coil of myosin⁵. These structures are stabilized by inter-strand hydrogen bonds, and their handedness is deter-

mined by the configuration of chiral centres in the nucleotide or peptide backbone. Synthetic hydrogen-bonded assemblies have been reported that display supramolecular chirality in solution^{6–8} or in the solid state^{9–12}. Complete asymmetric induction of supramolecular chirality—the formation of assemblies of a single handedness—has been widely studied in polymeric superstructures^{13,14}. It has so far been achieved in inorganic metal-coordinated systems^{15–17}, but not in organic hydrogen-bonded assemblies^{18–20}. Here we describe the diastereoselective assembly of enantio-pure calix[4]arene dimelamines and 5,5-diethylbarbituric acid (DEB) into chiral hydrogen-bonded structures of one handedness. The system displays complete enantioselective self-resolution: the mixing of homomeric assemblies (composed of homochiral units) with opposite handedness does not lead to the formation of heteromeric assemblies. The non-covalent character of the chiral assemblies, the structural simplicity of the constituent building blocks and the ability to control the assembly process by means of peripheral chiral centres makes this system promising for the development of a wide range of homochiral supramolecular materials or enantioselective catalysts.

Previously we have shown by X-ray crystallography and ¹H NMR spectroscopy that assembly 1₃•DEB₆ exclusively forms as the staggered isomer A (D₃), which displays supramolecular chirality both in solution and in the solid state (see Fig. 1)^{21,22}. In the absence of any other source of chirality, this isomer exists as a racemic mixture of the *M*- and *P*-enantiomers.

We have now found that assembly of 3 equivalents of the chiral calix[4]arene dimelamines (*R,R*)-2 or (*S,S*)-3, having (*R*)- or (*S*)-1-phenylethylamine moieties, respectively, with 6 equivalents of DEB gives quantitatively the assemblies 2₃•DEB₆ (*M*-enantiomer) or 3₃•DEB₆ (*P*-enantiomer) (compounds 1–7 are shown in Fig. 1). The induction of helicity in both assemblies is complete (d.e. > 98%, where d.e. is diastereomeric excess) as judged from the single set of signals in the ¹H NMR spectra (CD₂Cl₂) (Fig. 2). None of the other possible diastereoisomeric assemblies (that is, (*P*)-2₃•DEB₆ and (*M*)-3₃•DEB₆) is present. Two-dimensional rotating frame Overhauser effect spectroscopy (ROESY) experiments correlate the absolute configuration of the substituents with the helicity: an (*R*)-substituent induces *M*-helicity, and an (*S*)-substituent induces *P*-helicity, in the assembly (Fig. 2d). We note that both assemblies are strongly active in circular dichroism (CD; $\Delta\epsilon_{\max} \approx 100 \text{ cm}^2 \text{ mmol}^{-1}$, where $\Delta\epsilon_{\max} = (\epsilon_L - \epsilon_R)_{286 \text{ nm}}$, Fig. 3a), while none of the individual components (*R,R*)-2 or (*S,S*)-3 show any significant CD activity ($\Delta\epsilon_{\max} < 8 \text{ cm}^2 \text{ mmol}^{-1}$). The observed CD is thus clearly a direct result of assembly formation and not an intrinsic property of the individual components.

Complete induction of chirality was also observed for the (*S*)-alanine- and (*R*)-naphthyl dimelamine assemblies (*P*)-4₃•DEB₆ and (*M*)-5₃•DEB₆ (d.e. > 98% according to ¹H NMR spectroscopy; data not shown) and seems to be a general phenomenon for this type of assembly. Moreover, complete chiral induction is also observed with peripheral chiral centres in the cyanurate components. Combination of achiral dimelamine 6 with chiral cyanurates (*R*)- or (*S*)-MePhCYA or amino acid-derived cyanurates (*S*)-PheCYA, (*S*)-ValCYA, or (*S*)-LeuCYA (see Fig. 1 for nomenclature) leads in all cases to diastereoselective assembly of 6₃•CYA₆ with d.e. values > 98% according to ¹H NMR spectroscopy (data not shown). The CD spectra of the assemblies 2₃•DEB₆–5₃•DEB₆ and 6₃•CYA₆ all display bisignate curves with remarkably large amplitudes. The peripheral chromophores (benzyl, carbonyl, naphthyl) only affect the intensity of the Cotton effect (CE) at lower wavelengths, while the CD curves are virtually identical above 250 nm (Fig. 3). The observed Cotton effects seem to be largely the result of exciton coupling between chromophores present in the core of the assemblies. Comparison of the different CD spectra suggests that the sign of the CD curve is a good probe for the helicity of the assembly.

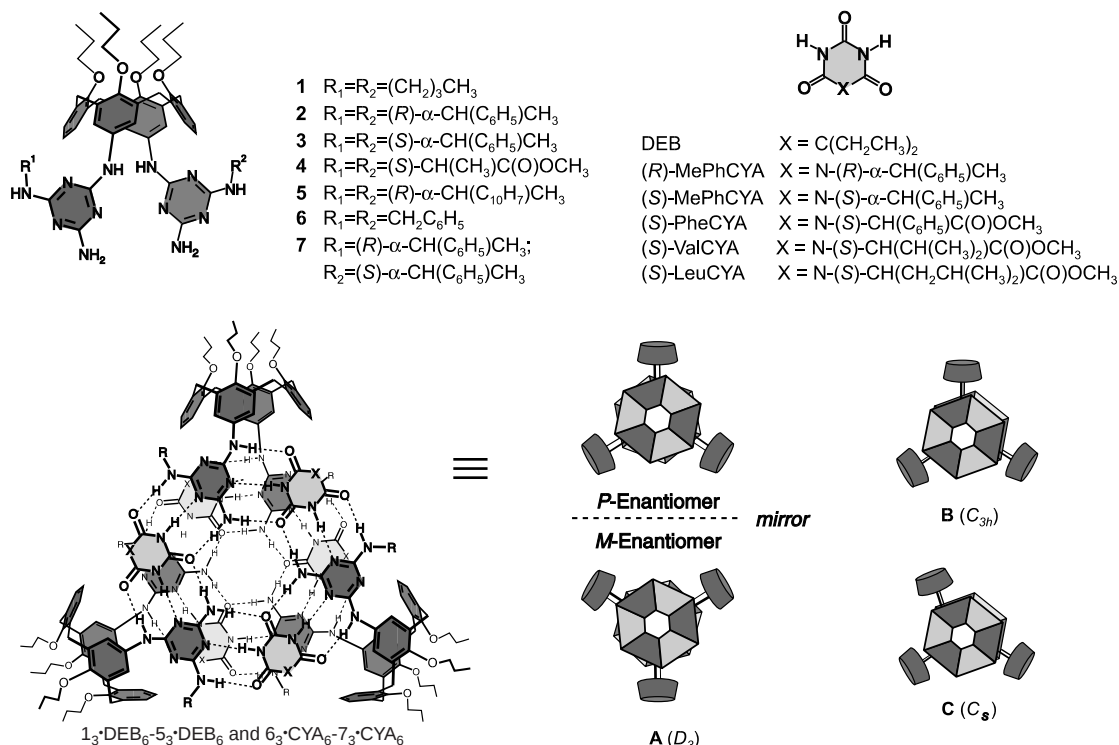


Figure 1 Schematic representations and molecular structure of the nine-component hydrogen bonded assemblies $1_3\bullet DEB_6-5_3\bullet DEB_6$, $6_3\bullet CYA_6$ and $7_3\bullet CYA_6$ with their individual components. The assemblies can exist in three isomeric forms: the chiral staggered isomer **A** (D_3 symmetry), and the achiral

eclipsed isomers **B** (C_{3h} symmetry) and **C** (C_s symmetry). The chiral isomer **A** can be present as a mixture of the *P*- and *M*-enantiomer. The assignment of the *M/P*-configuration is based on a clockwise (*P*) or anticlockwise (*M*) orientation of the two melamine units in the assembly²⁷.

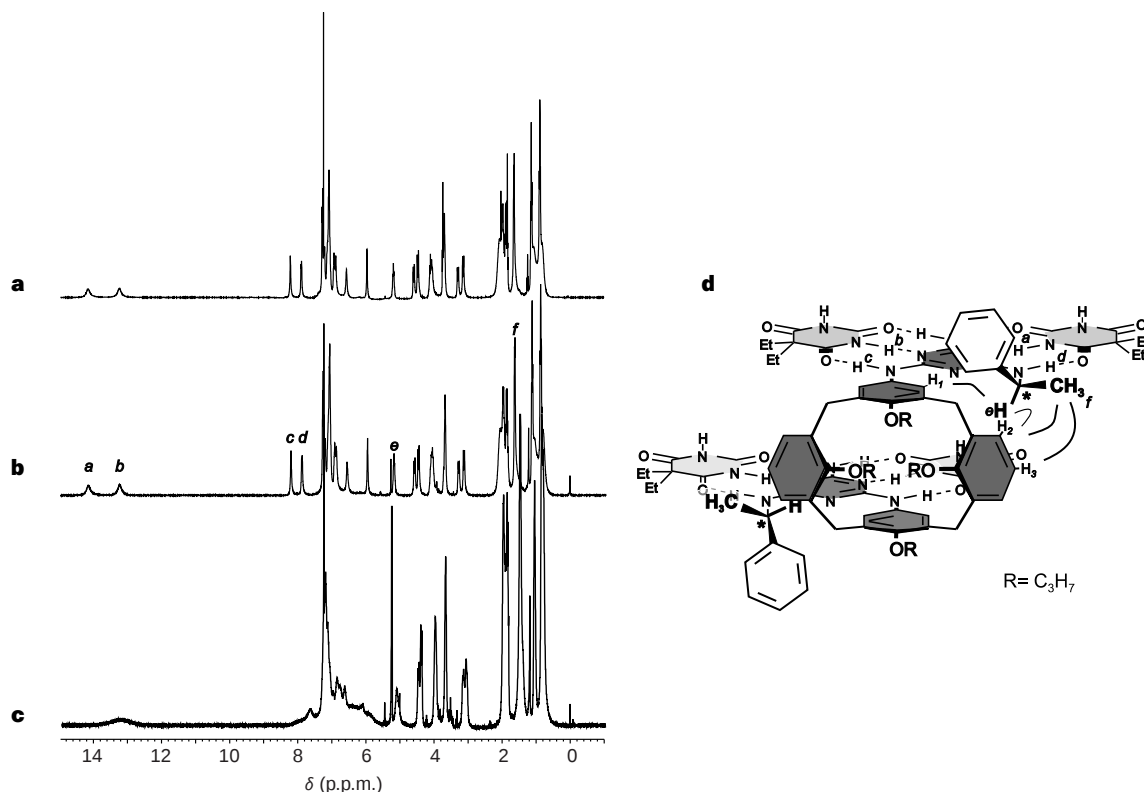


Figure 2 Characterization of chiral non-covalent assemblies (*M*)- $2_3\bullet DEB_6$ and (*P*)- $3_3\bullet DEB_6$ by 1H NMR spectroscopy. **a**, 400 MHz spectrum of enantiomerically pure assembly (*M*)- $2_3\bullet DEB_6$; **b**, 400 MHz spectrum of enantiomerically pure assembly (*P*)- $3_3\bullet DEB_6$; **c**, 400 MHz spectrum of the assembly of (*R,S*)-**7** and DEB (2 equiv.); all spectra were recorded in CD_2Cl_2 (1 mM) at 298 K relative to residual CH_2Cl_2 . **d**, 2D ROESY connectivities measured for assembly (*P*)- $3_3\bullet DEB_6$ in toluene- d_8 . Medium connectivities were observed for the following protons: H_a

and H_b , H_e and H_2 , H_f and H_2 , and H_f and H_3 . These connectivities relate the configuration of the chiral substituent (*S*) with the helicity (*P*) of the assembly. Additional evidence for the formation of assemblies (*M*)- $2_3\bullet DEB_6$ and (*P*)- $3_3\bullet DEB_6$ was obtained by MALDI-TOF mass spectrometry using Ag^+ -labelling²⁸. Both assemblies exhibit a strong signal at m/z 4,358.3 (calculated for $C_{234}H_{288}N_{48}O_{30}^{107}Ag^+$, 4,360.2) corresponding to the monovalent Ag^+ -complexes.

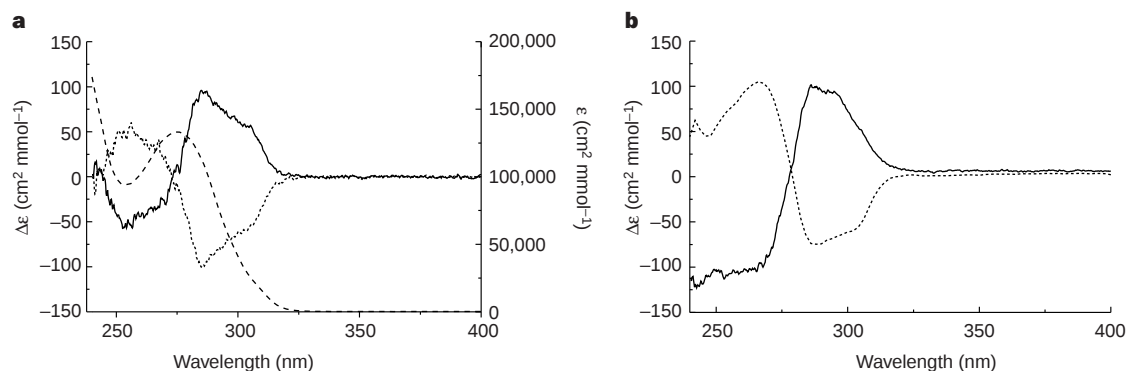


Figure 3 Characterization of assemblies $2_3\bullet\text{DEB}_6$ – $5_3\bullet\text{DEB}_6$ by CD and ultraviolet spectroscopy. **a**, CD spectra and ultraviolet spectrum (dashed line) of enantiomerically pure assemblies (M) - $2_3\bullet\text{DEB}_6$ (solid) and (P) - $3_3\bullet\text{DEB}_6$ (dotted); **b**, CD

spectra of enantiomerically pure assemblies (P) - $4_3\bullet\text{DEB}_6$ (dotted) and (M) - $5_3\bullet\text{DEB}_6$ (solid). Spectra were recorded in CH_2Cl_2 (1 mM) at 298 K.

The observed induction of supramolecular chirality is obviously related to the presence of six peripheral chiral centres in each assembly. The energy parameters for the chirality induction were determined using a theoretical model in which the difference in enthalpy between the M - and P -helix (ΔH_{total}) is regarded as the sum of six individual contributions of each chiral centre ($\Delta H_{\text{R/S}}$) (see Supplementary Information). Using this model, the values of ΔH_{total} and $\Delta H_{\text{R/S}}$ can be determined directly from the measured d.e. values via the relation: $\Delta H_{\text{total}} = 6\Delta H_{\text{R/S}} = -RT\ln[-(\text{d.e.}_{6\text{R}} + 100)/(\text{d.e.}_{6\text{R}} - 100)]$, where d.e._{6R} is the diastereomeric excess in an assembly with 6 (R)-substituents. For example, a d.e._{6R} > 98% gives $-\Delta H_{\text{total}}(298\text{ K}) > 11.4\text{ kJ mol}^{-1}$ and $-\Delta H_{\text{R/S}}(298\text{ K}) > 1.90\text{ kJ mol}^{-1}$. Cooperativity between the chiral centres is not included in the model, but will lead to the fact that $-\Delta H_{\text{R/S}} < -\Delta H_{\text{total}}/6$.

For a more accurate determination of $\Delta H_{\text{R/S}}$, we studied the heteromeric assemblies $2_{3-n}6_n\bullet\text{DEB}_6$ ($n = 1$ – 2), in which 1 or 2 chiral components (R,R -**2** are replaced by achiral components **6**. Determination of the corresponding d.e. values (d.e._{4R/2N} and d.e._{2R/4N})—which will be lower than d.e._{6R} as a result of the reduced number of chiral centres—will therefore allow a more accurate determination of $\Delta H_{\text{R/S}}$. The heteromeric assemblies $2_{3-n}6_n\bullet\text{DEB}_6$ ($n = 1$ – 2) cannot be obtained in pure form due to their dynamic character, but are formed spontaneously by mixing the homomeric assemblies (M) - $2_3\bullet\text{DEB}_6$ (Fig. 4a) and $6_3\bullet\text{DEB}_6$ (Fig. 4b), as indicated by multiple signals in the ^1H NMR spectrum (Fig. 4c). We have shown previously that mixtures of achiral assemblies have a

statistical composition²³. Titration of chiral assembly (M) - $2_3\bullet\text{DEB}_6$ with racemic assembly $6_3\bullet\text{DEB}_6$ followed by measurement of the CD spectra reveals a nonlinear relation between the chiroptical activity ($\Delta\epsilon_{286}$) of the mixture and the amount of added $6_3\bullet\text{DEB}_6$ (Fig. 4d). The chiroptical activity for each measurement is composed of three individual contributions: $\Delta\epsilon_{\text{total}} = (\text{d.e.}_{6\text{R}}[2_3\bullet\text{DEB}_6] + \text{d.e.}_{4\text{R}/2\text{N}}[2_26_1\bullet\text{DEB}_6] + \text{d.e.}_{2\text{R}/4\text{N}}[2_16_2\bullet\text{DEB}_6]) \times \Delta\epsilon(2_3\bullet\text{DEB}_6)$, assuming the same $\Delta\epsilon$ for each assembly $2_{3-n}6_n\bullet\text{DEB}_6$ ($n = 0$ – 3). Curve fitting (see Supplementary Information) using the theoretical model described above reveals the best fit for $-\Delta H_{\text{total}}(298\text{ K}) = 13.4\text{ kJ mol}^{-1}$ and $-\Delta H_{\text{R/S}}(298\text{ K}) = 2.23\text{ kJ mol}^{-1}$ (Fig. 4d), with corresponding d.e. values of 99.1% ($2_3\bullet\text{DEB}_6$), 94.7% ($2_26_1\bullet\text{DEB}_6$) and 71.7% ($2_16_2\bullet\text{DEB}_6$). The d.e._{6R} value of 99.1% for assembly (M) - $2_3\bullet\text{DEB}_6$ is in good agreement with that determined by ^1H NMR spectroscopy (see above). The chirality induction in the heteromeric assemblies $2_{3-n}6_n\bullet\text{DEB}_6$ ($n = 1$ – 2) resembles the ‘Sergeants and Soldiers’ principle (that is, the achiral units reinforce the induced chirality of the chiral units) as observed previously in polymeric²⁴ and liquid crystalline¹⁴ materials.

We also investigated mixtures of the chiral assemblies (M) - $2_3\bullet\text{DEB}_6$ and (P) - $3_3\bullet\text{DEB}_6$. The ^1H NMR spectrum of a 1:1 mixture of the assemblies is identical to that of the separate assemblies (Fig. 5a–c). Additional signals are not observed, which indicates that the heteromeric assemblies $2_{3-n}3_n\bullet\text{DEB}_6$ ($n = 1$ – 2) are not formed to a significant extent. Moreover, titration of (M) - $2_3\bullet\text{DEB}_6$ with (P) - $3_3\bullet\text{DEB}_6$ followed by CD shows a strictly

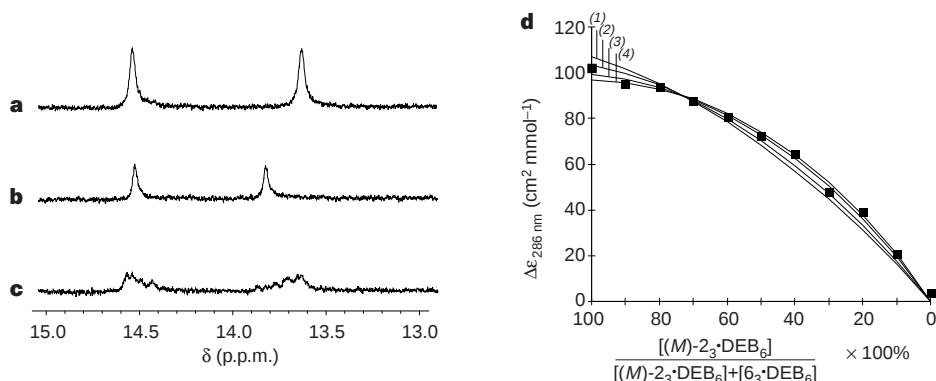


Figure 4 Characterization of mixed assemblies $2_{3-n}6_n\bullet\text{DEB}_6$ ($n = 0$ – 3) by ^1H NMR and CD spectroscopy. The figure shows the 13–15 p.p.m. region of the 300 MHz ^1H NMR spectrum of: **a**, enantiomerically pure assembly (M) - $2_3\bullet\text{DEB}_6$; **b**, racemic assembly $6_3\bullet\text{DEB}_6$; and **c**, a 1:1 mixture of (M) - $2_3\bullet\text{DEB}_6$ and $6_3\bullet\text{DEB}_6$. All spectra were recorded in toluene- d_8 (1 mM) at 298 K relative to residual

$\text{C}_6\text{D}_5\text{CHD}_2$. **d**, Plot of the CD intensity (filled squares, measured at 286 nm) versus the ratio $[(M)\text{-}2_3\bullet\text{DEB}_6]/[(M)\text{-}2_3\bullet\text{DEB}_6] + [6_3\bullet\text{DEB}_6] \times 100\%$. The lines represent the calculated curves for $\Delta H_{\text{total}} = -8.0(0)$, $-10.0(2)$, -13.4 (3, best fit) and -16.0 (4) kJ mol^{-1} .

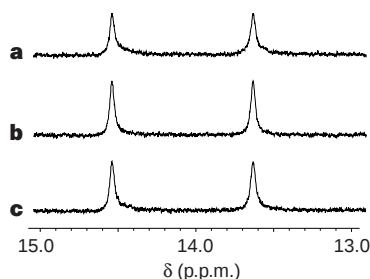
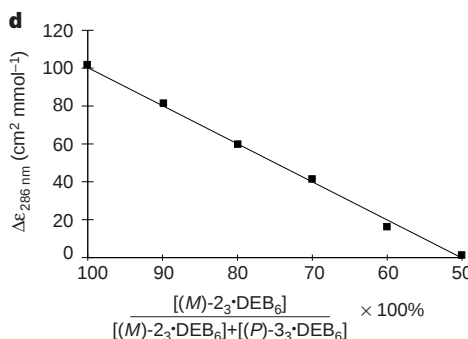


Figure 5 Characterization of mixed assemblies $2_3\text{-}n\text{-}3\text{-DEB}_6$ ($n = 0\text{--}3$) by ^1H NMR and CD spectroscopy. The figure shows the 13–15 p.p.m. region of the 300 MHz ^1H NMR spectrum of: **a**, enantiomerically pure assembly $(M)\text{-}2_3\text{-DEB}_6$; **b**, enantiomerically pure assembly $(P)\text{-}3_3\text{-DEB}_6$; and **c**, a 1:1 mixture of $(M)\text{-}2_3\text{-DEB}_6$ and



$(P)\text{-}3_3\text{-DEB}_6$. All spectra were recorded in toluene- d_8 (1 mM) at 298 K. **d**, Plot of the CD intensity (filled squares, measured at 286 nm) versus the ratio $[(M)\text{-}2_3\text{-DEB}_6]/[(M)\text{-}2_3\text{-DEB}_6] + [(P)\text{-}3_3\text{-DEB}_6] \times 100\%$. The solid line represents the calculated curve for $\Delta H_{\text{total}} = -15.0 \text{ kJ mol}^{-1}$.

linear decrease of the chiroptical activity (Fig. 5d). Curve fitting (see Supplementary Information) using the theoretical model reveals an improving fit for increasing $-\Delta H_{R/S}$ values. These results confirm the ^1H NMR experiment, and also indicate that the homomeric assemblies $(M)\text{-}2_3\text{-DEB}_6$ and $(P)\text{-}3_3\text{-DEB}_6$ are exclusively present in a mixture of the chiral building blocks $(R,R)\text{-}2$ and $(S,S)\text{-}3$ and DEB (3:3:6). In the case of statistical mixing, a 25:75 mixture of homomeric and heteromeric assemblies would be formed. The present system thus constitutes an example of complete enantioselective self-resolution in a dynamic hydrogen-bonded assembly in solution, a phenomenon so far only observed for hydrogen-bonded assemblies in the solid²⁵ or liquid-crystalline²⁶ state.

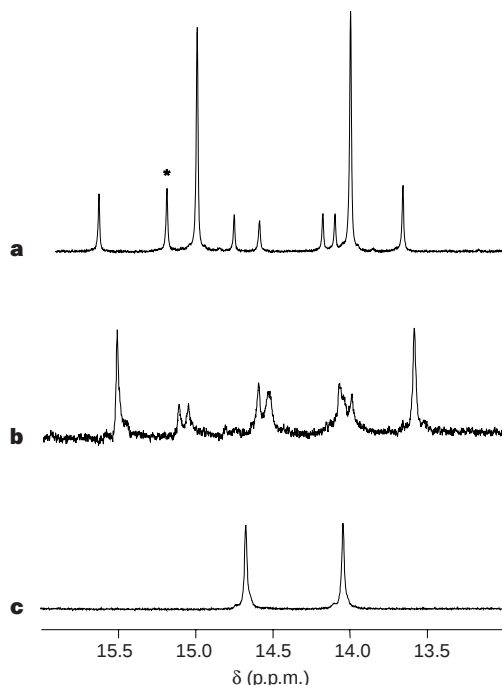


Figure 6 Isomeric distribution of hydrogen-bonded assemblies from **1**, $(R,R)\text{-}2$, and $(R,S)\text{-}7$ with HexCYA as determined by ^1H NMR spectroscopy. 300 MHz ^1H NMR spectrum (13–16 p.p.m.) of: **a**, assembly 1_3-HexCYA_6 (mixture of isomers **A**–**C**; the signal denoted with an asterisk represents two chemically different protons); **b**, *meso*-assembly 7_3-HexCYA_6 (mixture of isomers **B** and **C**); and **c**, enantiomerically pure assembly $(M)\text{-}2_3\text{-HexCYA}_6$ (exclusive formation of isomer **A**). Additional evidence for the formation of assemblies $(M)\text{-}2_3\text{-HexCYA}_6$ and 7_3-HexCYA_6 was obtained by MALDI-TOF mass spectrometry using Ag^+ -labelling²⁸. Both assemblies exhibit a strong signal at m/z 4,536.4 (calculated for $\text{C}_{240}\text{H}_{308}\text{N}_{54}\text{O}_{30}\text{-}^{107}\text{Ag}^+$, 4,535) corresponding to the monovalent Ag^+ -complexes.

Chiral information in the individual components can also be used to control the conformation of the assembly. As we have shown above, both $(R,R)\text{-}2$ and $(S,S)\text{-}3$, which have two chiral centres of identical configuration, preferentially assemble with DEB as the staggered isomer **A** (Fig. 1). In sharp contrast to this, dimelamine $(R,S)\text{-}7$, which has two chiral centres of opposite configuration, is not able to form isomer **A** in the presence of 2 equivalents of DEB. In this case, non-defined oligomeric assemblies are formed that show broad signals in the ^1H NMR spectrum (Fig. 2c). More informative are the results obtained from assembly studies of **1**, $(R,R)\text{-}2$, and $(R,S)\text{-}7$ with HexCYA. Assembly 1_3-HexCYA_6 exists as a mixture of isomer **A** (D_3) and the two achiral eclipsed isomers **B** (C_{3h}) and **C** (C_s) (Fig. 1). The ^1H NMR spectrum exhibits 10 different signals in the 14–16 p.p.m. region; that is, two signals each for **A** and **B**, and six signals for **C** (Fig. 6a). However, for $(R,R)\text{-}2$ and $(R,S)\text{-}7$ the isomeric distribution of the assembly with HexCYA is changed dramatically. Assembly of 3 equivalents of $(R,S)\text{-}7$ with 6 equivalents of HexCYA gives a mixture of the two eclipsed isomers **B** and **C**. The ^1H NMR spectrum shows only eight signals in the 14–16 p.p.m. region, and isomer **A** is not present (Fig. 6b). In contrast to this, assembly of 3 equivalents of $(R,R)\text{-}2$ and 6 equivalents of HexCYA gives isomer **A** as the only product. Not a trace of the isomers **B** and **C** is found in the ^1H NMR spectrum; there are only two signals in the 14–16 p.p.m. region (Fig. 6c). Assembly 2_3-HexCYA_6 exhibits a CD spectrum characteristic of assemblies of the isomer **A** type. These experiments again illustrate the decisive role that the peripheral chiral centres play in the assembly process. □

Methods

Hydrogen-bonded assemblies were prepared by suspending calix[4]arene dimelamines **1**–**7** and either DEB or CYA in a 1:2 molar ratio in toluene or CH_2Cl_2 . Clear solutions were obtained after stirring the mixtures for 15 min at room temperature. Occasionally sonication or heating was required to dissolve the components. For the CD-titration experiments 1 mM solutions of the homomeric assemblies in CH_2Cl_2 were mixed in ratios of 10:1 to 1:10 for $(M)\text{-}2_3\text{-DEB}_6$ and 6_3-DEB_6 and 10:1 to 5:5 for $(M)\text{-}2_3\text{-DEB}_6$ and $(P)\text{-}3_3\text{-DEB}_6$. The CD spectra were recorded 15 min after mixing the separate solutions.

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Variability of inorganic and organic phosphorus turnover rates in the coastal ocean

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Phosphorus is an essential nutrient in pelagic marine ecosystems. Phosphorus cycling in the upper ocean is, however, poorly understood, and few studies have directly investigated the biological utilization of this essential element^{1–4}. Here, we have determined *in situ* phosphorus-turnover rates in a coastal marine environment by measuring the activities of two cosmogenic radionuclides (^{32}P and ^{33}P , with half lives of 14.3 and 25.3 days, respectively) in dissolved inorganic, dissolved organic and total particulate phosphorus pools over a seasonal cycle. Phosphorus turnover rates within dissolved and particulate pools are rapid and vary over seasonal timescales, suggesting that low phosphorus concentrations can support relatively high primary production. Furthermore, picoplankton, such as bacteria, appear preferentially to

utilize certain dissolved organic phosphorus compounds to obtain other associated nutrients, such as carbon and nitrogen. It seems that the significance of the roles of both dissolved inorganic and organic phosphorus in supporting primary production—and, hence, CO_2 uptake and particulate organic carbon export—has been hitherto underestimated.

The radionuclides ^{32}P and ^{33}P are produced primarily by cosmic ray interactions with atmospheric argon and enter the oceans predominantly in rain^{5–7}. If the ratio of $^{33}\text{P}/^{32}\text{P}$ in rain is known, then one can determine the relative ‘age’ of cosmogenic phosphorus, P, by measuring the $^{33}\text{P}/^{32}\text{P}$ ratio in various biological pools. High $^{33}\text{P}/^{32}\text{P}$ ratios indicate an older P pool. The inventories of ^{32}P and ^{33}P in the ocean are quite low, ranging from just tens to hundreds of disintegrations per minute per square metre (d.p.m. m^{-2})^{1,8–10}. Thus, ^{32}P and ^{33}P measurements require several thousand litres of sea water and extensive purification from other β -emitters. Previous investigations which sought to utilize these isotopes were hampered by a lack of known input fluxes, possible contamination and the inability to measure the low-energy β -emitter ^{33}P , especially in coastal environments with high P concentrations^{1,8–10}. This study is, to our knowledge, the first to constrain the ^{32}P and ^{33}P input flux⁶ and to simultaneously measure both these isotopes in various dissolved inorganic, organic and particulate pools.

Sampling was conducted in Wilkinson basin in the Gulf of Maine ($42^\circ 29.41' \text{N}$, $69^\circ 45.02' \text{W}$) during four cruises in March, April, July and August 1997. This highly productive region supports one of the largest fisheries in North America¹¹. Surface and deep particulate and total dissolved phosphorus (TDP) samples were collected by passing 4,000–6,000 l of sea water sequentially through a series of 10, 1.0 and 0.2 μm cartridge prefilters followed by cartridges packed with iron-impregnated polypropylene filters. These filters have been demonstrated to collect TDP with close to 100% efficiency⁷. Separate surface samples were collected for soluble reactive phosphorus (SRP), as defined by the molybdenum blue method¹², using acrilan filters and the technique developed by Lee *et al.*⁹. Plankton tows (nominally $>102 \mu\text{m}$) were collected from various depths and sieved through a 335- μm screen to collect different size classes. All samples were extensively purified to remove all other β -emitting radionuclides and counted using low-level liquid scintillation⁶. In March and April, deep-water P samples were taken just above the base of the mixed layer (defined by a change in density $\geq 0.125 \text{ kg m}^{-3}$), whereas in July and August, deep-water samples were taken below both the mixed layer and the deep chlorophyll maximum.

The ratio of $^{33}\text{P}/^{32}\text{P}$ measured in rain at Portsmouth, NH and Woods Hole, MA was flux weighted over a 35 ± 3 -day period before the April, July and August cruises and over a 14-day period before the March cruise. $^{33}\text{P}/^{32}\text{P}$ ratios averaged 0.82 ± 0.07 (Fig. 1, Table 1). Thus, any ratio higher than this value must be due to radioactive decay. A non-continuous model can be used to determine the relative age of phosphorus in any particular reservoir: $\tau_p = [\ln(R_p/R_s)]/(\lambda_{32} - \lambda_{33})$ where τ_p is the age of phosphorus in the product material, R_p and R_s are the $^{33}\text{P}/^{32}\text{P}$ ratio found in the product and source material, respectively, and λ_{32} and λ_{33} are the radioactive decay constants⁵. Using this model, phosphorus ages are resolved on timescales ranging from 1 to 100 days. In general, age estimate errors will increase with increasing $^{33}\text{P}/^{32}\text{P}$ ratios as P activities decrease over time.

In March, April and July, ratios of $^{33}\text{P}/^{32}\text{P}$ in particulate matter were similar between surface and deep waters, indicating rapid transport of sinking particulate material from the euphotic zone to depth (Fig. 1). In August, the activities in all particulate samples retrieved from deep waters were below detection, indicating that the source of sinking particulates had decreased. $^{33}\text{P}/^{32}\text{P}$ ratios in total dissolved and small particulate ($<102 \mu\text{m}$) surface pools measured during March, April and August were similar to those found in rain.

Table 1 ^{32}P and ^{33}P inventories from rain and seawater measurements

Sample	Inventory expected from measured rain input (d.p.m. m ⁻²)				Measured ³² P† seawater inventory (d.p.m. m ⁻²)	Measured ³³ P† seawater inventory (d.p.m. m ⁻²)
	³² P		³³ P			
	NH*	MA†	NH*	MA†		
March	73	168	98	236	204	206
April	72	272	98	435	157	142
July	122	54	148	84	89	89
August	133	66	196	129	59	67

* NH represents Portsmouth, New Hampshire (43° 04' N, 70° 42' W).

† MA represents Woods Hole, Massachusetts (41° 32' N, 70° 39' W).

‡ Over upper 50 m at Wilkinson basin.

This indicates rapid P turnover rates on timescales of less than a day to just over a week (Figs 1 and 2a, b, and Supplementary Information). In July, however, the age of TDP increased to two weeks (Fig. 2a).

In July and August, the separation of TDP and SRP allowed for the estimation of the activity of both ^{32}P and ^{33}P in dissolved organic phosphorus DOP (= TDP – SRP). In contrast to previous investigations^{1,5,7–9}, these results demonstrate the *in situ* temporal variability in the ^{32}P and ^{33}P activity and the P residence time of DOP. Surface DOP concentrations increased from a low of 0.04 μM in April to 0.20 μM in July, whereas SRP concentrations decreased from 0.56 to 0.26 μM . In July, the relative age of the DOP pool was 28 days and the activity of ^{32}P and ^{33}P in the DOP pool was close to 50% of the measured ^{32}P and ^{33}P TDP activity (Fig. 2a). In contrast, the relative fraction of ^{32}P and ^{33}P activity in the August DOP pool was over a factor of two lower, whereas DOP concentrations decreased by less than 5%. The measured decrease in August DOP activity, however, could not be accounted for by radioactive decay alone. This implies that there was preferential remineralization of a small fraction of DOP compounds that were younger than the bulk DOP pool. The remaining DOP fraction must cycle over timescales longer than 100 days.

Concurrent with the decrease in DOP activity was an increase by a factor of five in both ^{32}P and ^{33}P activities within the picoplankton (0.2–1.0 μm) size class. Assuming that all the P uptake of the 0.2–1.0- μm -size class was derived from the TDP pool, a picoplankton P turnover rate of approximately two days can be determined for August (Fig. 2b). This is comparable to a bacterial P residence time of several hours to several days found by previous investigations using incubation techniques^{2,3}.

The relatively high ^{32}P and ^{33}P activities and low $^{33}\text{P}/^{32}\text{P}$ ratio found within the August picoplankton (0.2–1.0 μm) size class also suggests that these organisms were consuming SRP and possibly small particulate P (1.0–10 μm and 10–102 μm). Although low in ^{32}P and ^{33}P activity, rapid remineralization of the 0.1–10- μm and 10–102- μm P pools could support a significant fraction of the bacterial P uptake. Whereas previous studies have shown SRP and particulate P uptake in incubation experiments^{13–15}, our results demonstrate the rapidity of these processes in the water column.

During uptake, P is incorporated into a wide variety of biological components. However, the rate of incorporation and the distribution of this uptake will vary depending on the environment and growth cycle of the organism. Preferential remineralization of DOP compounds with relatively high ^{32}P and ^{33}P activities suggest that these compounds were more bioavailable than the bulk DOP pool. Previous research has indicated that the proportion of phosphonates within the high molecular weight (1–100 nm) fraction of DOP was significantly higher than that found in natural assemblages of plankton¹⁶. Thus, it was hypothesized that the remineralization of other DOP compounds was preferred. Several studies have found that bacteria can readily hydrolyse organic phosphorus compounds, such as monophosphate esters and nucleotides, through enzymatic processes^{4,17–19}. Our results suggest that bacteria are rapidly remineralizing these compound classes. If correct, our results also

imply that phytoplankton rapidly incorporate P from outside the cell into nucleotides and monophosphate esters. These compounds are then released through grazing or virolytic processes, thereby providing a mechanism in which more bioavailable DOP compounds can contain higher ^{32}P and ^{33}P activities than the bulk DOP pool. Regardless, these results provide direct evidence that there is a more bioavailable pool of DOP.

The observation that SRP is also being consumed indicates that the picoplankton-size organisms must be expending energy in the hydrolysis of DOP for reasons other than P limitation. Dissolved organic carbon concentrations in surface waters decreased from 78.2 μM in July to 64.5 μM in August, while combined nitrate and nitrite levels averaged 0.1 μM . Thus, we hypothesize that bacteria and picoplankton were hydrolysing specific DOP compounds for their carbon (C) and possibly nitrogen (N) content, presumably as structural biosynthetic precursors.

These results have several important implications concerning the P cycle in the open ocean. Our results demonstrate that the

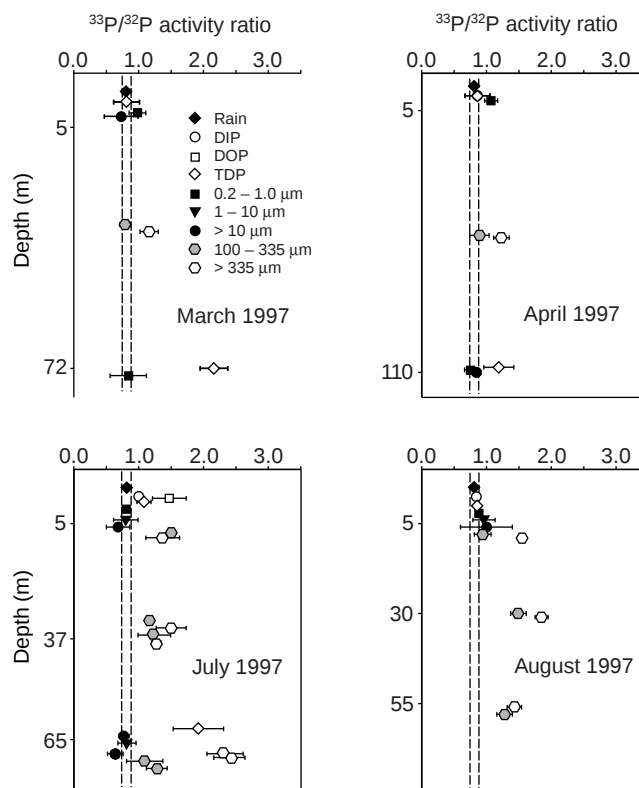


Figure 1 $^{33}\text{P}/^{32}\text{P}$ ratios in rain and sea water. Seawater samples were obtained during March, April, July and August 1997 cruises in the Gulf of Maine. With the exception of the March and April plankton tows, samples were taken at the specific depth listed along the y-axis. Vertical separation is for clarity. In March and April, plankton tows were integrated over the depth of the mixed layer and are shown at the midpoint of depth collection.

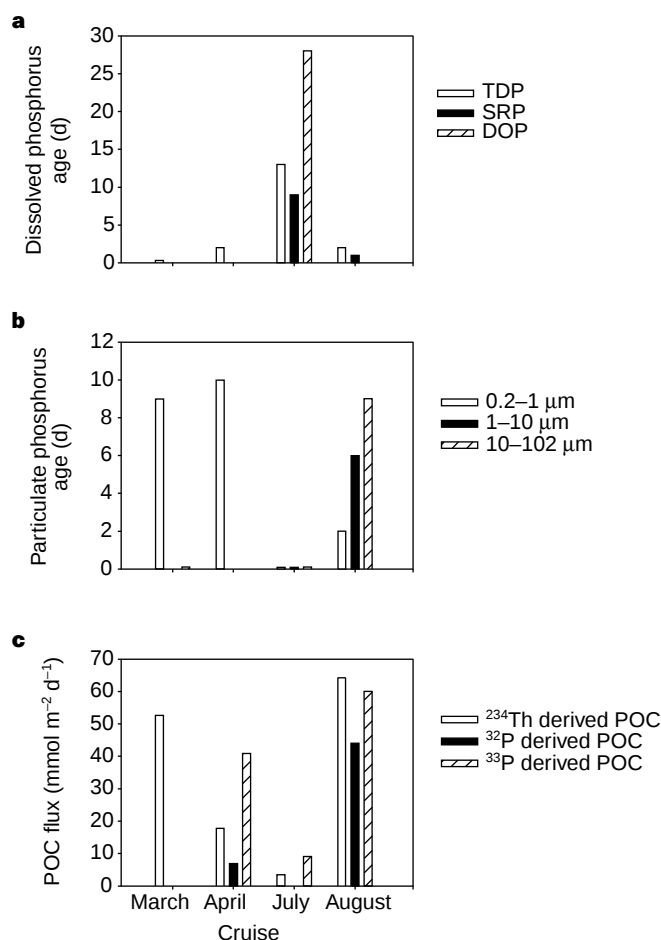


Figure 2 Phosphorus ages and particulate organic carbon (POC) fluxes. **a**, Dissolved phosphorus ages. Separate SRP samples were not taken during the March and April cruises. In August, a DOP age could not be determined owing to the large errors associated with the P activity measurements. **b**, Small particulate phosphorus ages. Bars not shown had P activities below detection. In April, the 0.2–1.0 µm and 1.0–10 µm fractions were combined before measurement. **c**, Radionuclide-derived POC export. Methods for the ³²P and ³³P derived POC fluxes are described in the text. POC fluxes derived from ²³⁴Th were taken from the same cruises according to the methods described by Buesseler²².

residence times of SRP and DOP are distinct from one another and vary seasonally, suggesting that these two pools play distinct roles in biological production. Even at low concentrations, rapid turnover of SRP can support a much greater fraction of primary production than previously thought. This has significant implications for those studies that assume P limitation based only on P concentration (relative to N). In addition, bacteria and picoplankton can obtain not only additional P, but also C and N through the breakdown of DOP. The bioavailable DOP pool, however, represents a small fraction of bulk DOP within coastal environments. Nonetheless, picoplankton in the presence of DOP may substantially increase primary production in the open ocean.

In contrast to the 0.2–1.0 µm size class, ³²P and ³³P activities in the 1–10 µm and 10–102 µm size fractions had decreased by more than 50% between July and August, and the average residence time increased from less than two days to just over a week (Fig. 2b). This implies that both autotrophic and heterotrophic P consumption had dropped. Given the available SRP concentrations, a decrease in phytoplankton activity suggests limitation by trace metals and/or other nutrients. The reduction in heterotrophic activity, however, indicates that microzooplankton, such as ciliates, were inefficient grazers of picoplankton. This is in direct contrast to laboratory

studies which have found that microflagellate grazing plays a dominant role in both trace-metal and nutrient recycling under oligotrophic conditions^{20,21}. Furthermore, although picoplankton can increase primary production, an increase in secondary production and particulate export does not always result.

Particulate export fluxes over the upper 50 m were determined for ³²P and ³³P assuming steady state and negligible advection and diffusion, as well as considering the difference between the average rain flux⁶ at Woods Hole (Massachusetts) and Portsmouth (New Hampshire) and the measured seawater inventory (Table 1). Although ³³P/³²P ratios did not vary significantly between the two sites, fluxes varied by as much as a factor of four. These large flux variations are most likely to be due to differences in the source of the scavenged air mass⁷. Because rainfall was not measured directly at our seawater sampling site, an average value between the two rain stations was used to determine the input of ³²P and ³³P. In March, the Portsmouth data were limited and suffered from rain-gauge inaccuracies associated with large snowfall⁷. As a result, ³²P and ³³P export fluxes could not be determined for the March cruise.

The flux of particulate organic carbon (POC) was found by multiplying the calculated radionuclide fluxes by the ratio of ³²P (and ³³P) to carbon on particulates (≥1 µm). Using an average ³²P and ³³P rain input gave POC fluxes which agreed well with those determined using the more commonly utilized²² radioisotope POC flux tracer, ²³⁴Th (Fig. 2c). Using an annual Gulf of Maine primary production estimate of 66.2 mmol C m⁻² d⁻¹ (from 1978–1980)¹¹ allows the calculation of an average ‘export’ ratio of 0.52. Given the low activities of ³²P and ³³P observed in the 1–10 µm and 10–102 µm size fractions, it is likely that the large increase in carbon export observed during the late summer is the result of zooplankton growth in July and mortality in August (Fig. 2c). This further indicates a lag period between changes in community structure and organic carbon export.

³²P and ³³P activities within the larger microplanktonic pools (>102 µm) were an order of magnitude lower than those found in the smaller particles (<102 µm). In March and April, both the 102–335 µm and the >335-µm size classes were dominated by phytoplankton and had turnover rates which ranged from a day to as long as two weeks. In July and August, residence times of particles in the 102–335-µm class were longer, ranging from two to four weeks (mixed assemblages of copepods and dinoflagellates). The >335-µm size class, however, had residence times as long as seven weeks (dominated by mature copepods). These P residence time estimates are similar to the 19 days found by laboratory studies which fed copepods with ³²P-labelled phytoplankton²³ and to the 30–80 day range found in plankton tows using *in situ* measurements of ³²P and ³³P at Bermuda and off the coast of Southern California^{1,8,10}. However, our study is one of the first to show a seasonal relationship between P ages and trophic level. Our results provide additional evidence that increasing primary production within the Gulf of Maine does not immediately result in an increase in macrozooplankton P uptake.

The measurement of ³²P and ³³P within dissolved and particulate pools has provided much-needed insight into the temporal variability of P cycling in the upper ocean. Further measurements of these isotopes, especially in regimes that appear to be P limited, will help to provide new constraints on models that seek to emulate primary production and particulate export. It is already clear that ³²P and ³³P have the unique ability to pinpoint the most biologically ‘active’ pools of dissolved inorganic, organic and particulate P pools. □

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Large-scale impoverishment of Amazonian forests by logging and fire

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Amazonian deforestation rates are used to determine human effects on the global carbon cycle^{1–3} and to measure Brazil's progress in curbing forest impoverishment^{1,4,5}. But this widely used measure of tropical land use tells only part of the story. Here we present field surveys of wood mills and forest burning across

Brazilian Amazonia which show that logging crews severely damage 10,000 to 15,000 km² yr⁻¹ of forest that are not included in deforestation mapping programmes. Moreover, we find that surface fires burn additional large areas of standing forest, the destruction of which is normally not documented. Forest impoverishment due to such fires may increase dramatically when severe droughts provoke forest leaf-shedding and greater flammability; our regional water-balance model indicates that an estimated 270,000 km² of forest became vulnerable to fire in the 1998 dry season. Overall, we find that present estimates of annual deforestation for Brazilian Amazonia capture less than half of the forest area that is impoverished each year, and even less during years of severe drought. Both logging and fire increase forest vulnerability to future burning^{6,7} and release forest carbon stocks to the atmosphere, potentially doubling net carbon emissions from regional land-use during severe El Niño episodes. If this forest impoverishment is to be controlled, then logging activities need to be restricted or replaced with low-impact timber harvest techniques, and more effective strategies to prevent accidental forest fires need to be implemented.

Human uses of tropical forests vary greatly in their ecological impacts. Ranchers and farmers 'deforest' land in preparation for cattle pasture and crops by clear-cutting and burning patches of forest. Loggers do not clear-cut and burn, but perforate forests by harvesting or damaging many trees. Rubber tapping and similar activities use the forest at very low intensity through the harvest of animals, fruits, latex and other "non-timber products"^{8–11}. Deforestation by ranchers and farmers has a greater effect on forest carbon content, forest hydrology, and the diversity of native plant and animal species than other forest uses^{9–14} and has become the main parameter by which human effects on tropical forests are measured. Part of the appeal of this forest versus non-forest approach to assessing human effects on tropical forests is its tractability. Forest conversion to agriculture is readily monitored from space using imagery from the Landsat Thematic Mapper (TM) satellites, permitting the development of deforestation maps of large regions at a reasonable cost and speed^{4,5}.

This binary approach to the analysis of human effects on tropical forests neglects those forest alterations that reduce tree cover, but do not eliminate it, such as logging and surface fires in standing forests. The forest openings created by logging and accidental surface fires are visible in Landsat TM images, but they are covered over by regrowing vegetation within 1 to 5 years, and are easily misclassified in the absence of accompanying field data¹⁵. Although logging and forest surface fires usually do not kill all trees, they severely damage forests. Logging companies in Amazonia kill or damage 10–40% of the living biomass of forests through the harvest process^{9,10,16}. Logging also increases forest flammability by reducing forest leaf canopy coverage by 14–50%^{7,9,10,16,17}, allowing sunlight to penetrate to the forest floor, where it dries out the organic debris created by the logging. Fires ignited on agricultural lands can penetrate logged forests^{7,17,18}, killing 10–80% of the living biomass^{6,17} and greatly increasing the vulnerability of these forests to future burning^{6,19}. Fires from agricultural lands can also penetrate those undisturbed forests that have lost portions of their leaf canopies because of severe seasonal drought¹⁹.

We estimated the area of Brazilian Amazonian forest that is impoverished each year through logging by interviewing 1,393 wood mill operators, representing more than half of the mills located in 75 logging centres (Table 1); these logging centres are responsible for >90% of Amazonian timber production. In each interview, we obtained the mill's harvest records of roundwood (tree trunks) for 1996 and 1997 and the harvest rate (m³ of timber per ha of forest), thereby calculating the forest area required to supply each centre's timber production. The accuracy of the roundwood harvest rates reported by mill operators was tested by comparing these interview data with direct measurements of roundwood harvest in

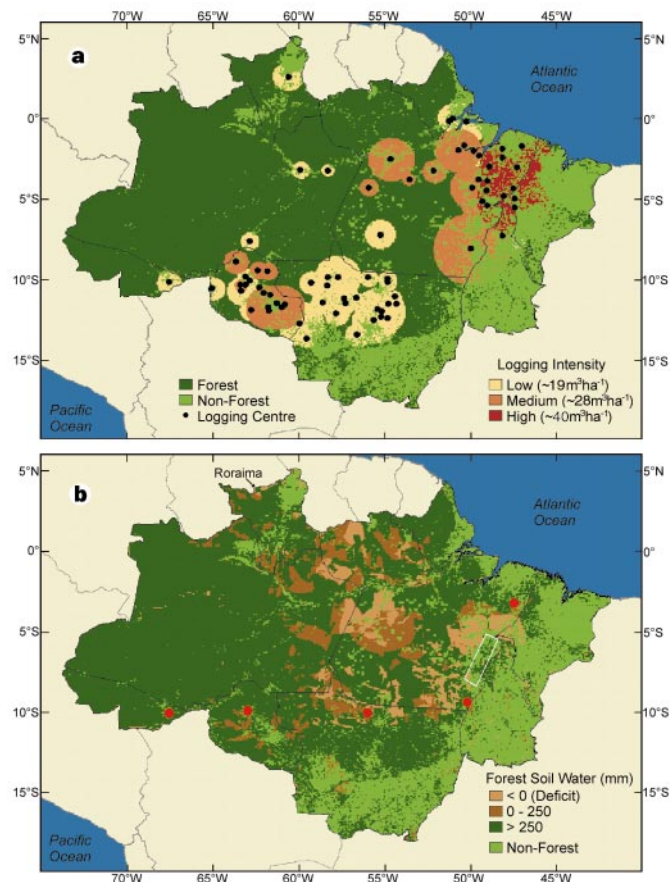


Figure 1 Patterns of forest logging and severe soil water deficits. **a**, Forest regions in Brazilian Amazonia that lie within the average extraction distances of 75 logging centres which account for >90% of Amazonian timber production. Extraction intensities are 'low' (19 m³ ha⁻¹), 'moderate' (28 m³ ha⁻¹) and 'high' (40 m³ ha⁻¹). State boundaries of the Brazilian Amazon and national boundaries of neighbouring countries are displayed. **b**, Estimated forest plant-available soil water content to five metres depth, at the end of the 1998 dry season (30

September). Forests with a soil water deficit (<0 mm) had depleted all plant-available water in the upper 5 m of soil, and we predict that they were highly vulnerable to fire. Forests with <250 mm of plant-available soil water may have also been vulnerable to fire. Red dots indicate locations of landholder interviews, from west to east: Rio Branco, Acre; Ariquemes, Rondônia; Alta Floresta, Mato Grosso; Santana do Araguaia, Pará; and Paragominas, Pará. The white rectangle indicates the region of the aeroplane study of forest fire occurrence.

~100-ha forest plots that had been harvested at low ($n = 12$) (ref. 9, and A.V., E.L., R. Júnior and C. Leão, unpublished report), moderate ($n = 7$, unpublished data, Tropical Forest Foundation) and high ($n = 3$)¹⁰ harvest intensities. In each comparison, harvest rates measured directly in the forest were within the 95% confidence interval of the average harvest rates reported by mill operators in the nearest logging centre. Mahogany mills were excluded from this study because their immediate effect on the forest is very small compared to other types of logging, and the volume of mahogany

extraction is <5% of total Amazonian production (although mahogany extraction may encourage additional human activities in forests)¹⁶.

The area of standing forest subjected to surface fire each year was assessed by interviewing 202 landholders in five regions along a 2,200-km transect through the states of Pará, Mato Grosso, Rondônia and Acre. The properties had a total area of 9,200 km², and were randomly selected within each of four size categories. In each interview, the landholders drew onto satellite images the forest areas

Table 1 Roundwood production, logging intensity and rates (1996–97), and deforestation (1997) in the Brazilian Amazon

State	Total no. of logging centres	Total no. of mills	Mills studied (%)	Roundwood production* (10 ⁶ m ³)	Intensity of logging† (% of production)			Forest area affected (km ² yr ⁻¹)			Original forest area [‡] (km ²)
					Low	Med.	High	Logging‡ 1996–97	Deforestation [‡]		
									1993–95	1996	
Acre	1	25	55	0.3	100	0	0	120–210	720	430	152,394
Amapa	2	89	80	0.2	100	0	0	80–140	0	0	137,444
Amazonas	3	20	60	0.7	100	0	0	290–500	950	1,020	1,531,122
Maranhão	2	52	49	0.7	0	0	100	160–200	830	1,060	145,766
Mato Grosso	22	708	48	9.8	100	0	0	4,080–7,000	7,610	6,540	527,570
Pará	24	1,324	43	11.9	11	61	28	3,560–4,910	5,470	6,130	1,183,571
Rondônia	19	272	55	3.9	25	75	0	1,320–1,920	3,310	2,430	212,214
Roraima	1	25	52	0.2	100	0	0	80–140	230	210	172,425
Tocantins	1	18	53	0.1	100	0	0	40–70	490	320	30,325
Total	75	2,533	48	27.8	49	41	10	9,730–15,090	19,610	18,140	4,092,831

* Logging centres, by our definition, produce at least 100,000 m³ of roundwood each year, and are responsible for >90% of Brazilian Amazonian roundwood production.

† Low-intensity logging, 19 (14–24) m³ ha⁻¹; moderate intensity, 28 (24–32) m³ ha⁻¹; high intensity, 40 (35–45) m³ ha⁻¹. Values here are mean (95% confidence interval).

‡ See text for definition.

on their property that had been deforested and the forest areas that had burned by surface fire (without prior forest felling), in 1994 and 1995. The accuracy of these landholder maps was tested by comparing them, in one study region, with a 1995 Landsat TM image. Forest surface fire scars were identified within the image by analysis of spectral characteristics, and areas of deforestation were identified visually. Within the 640 km² test area, all of the forest surface fires and deforestation burns reported by landholders were detected in the Landsat image. However, the landholders underestimated the area of surface fires by 43% and of deforestation by half.

We estimate that 10,000–15,000 km² of undisturbed forest were logged per year in 1996 and 1997 by the 2,533 wood mills operating in Brazilian Amazonia, based on the 95% confidence interval of the harvest intensities reported by mill operators. Hence, annual logging in these years affected a forest area that was 50–90% the size of the area that was completely deforested in 1996⁴ (Table 1). Virtually all of the forests of eastern Amazonia lie within the average harvest distance of logging centres in Pará and Maranhão states, and are being harvested at high (40 m³ ha⁻¹) and moderate (28 m³ ha⁻¹) intensities (Fig. 1a).

Within properties surveyed in the fire study, the area of standing forest that was affected by surface fire in 1994 and 1995 (310 km²) was 1.5 times greater than the area that was deforested in those years (200 km²). Most of the forests that experienced surface fire in Paragominas and Rondônia had already been logged, but large areas of undisturbed forest burned in Santana do Araguaia and Mato Grosso. Although extrapolation of this data set to the entire Amazon is not warranted, these data indicate that the area of Amazon forest affected by surface fire each year may be similar in scale to the area affected by deforestation.

The area of forest surface fires may be much larger during periods of severe drought, such as occurred during the 1997–98 El Niño/Southern Oscillation (ENSO) episode when 15,000 km² of standing forest may have burned in the northern Amazonian state of Roraima alone (Brazilian Government, unpublished report). We assessed the potential for large-scale, drought-induced Amazonian forest burning as a result of this ENSO episode using a regional water balance model. The model tracks soil water beginning on 1 May 1997, the end of the 1997 rainy season, when we assume that the soil was fully charged with water. Amazonian forests can tap the water stored in deep soil layers to maintain evapotranspiration during periods of low rainfall^{13,20}. We assume that forests become flammable only when soil moisture is depleted to five metres depth, based on field studies of soil moisture, leaf shedding¹³, fine fuel moisture and the propagation of experimental fires (D.C.N., M.C., E.M., F. Brown & J. Guerreros, unpublished data). The maximum amount of plant-available water that can be stored in the soils was calculated for the forested areas of Brazilian Amazonia using soil texture data from 1,147 soil profiles^{21–23}. Rainfall data from 1 May 1997 to 30 September 1998 were obtained from 30 to 60 automated weather stations scattered across Brazilian Amazonia (Instituto Nacional de Pesquisas Espaciais, unpublished data), which also provided air temperature data that we employed to estimate evapotranspiration using a corrected Thornwaite equation²⁴. These estimates of evapotranspiration are within 5% of values measured in an eastern Amazon forest²⁰.

ENSO-related drought can desiccate large areas of Amazonian forest, creating the potential for large-scale forest fires. Because of the severe drought of 1997 and 1998, we calculate that approximately 270,000 km² of Amazonian forest had completely depleted plant-available water stored in the upper five metres of soil by the end of the 1998 dry season. In addition, 360,000 km² of forest had less than 250 mm of plant-available soil water left by this time (Fig. 1b). By comparison, only 28,000 km² of forests in Roraima had depleted soil water to 5 m depth at the peak of the Roraima forest fires. We estimated the areal extent of forest surface fire in a 45,000-km² southeastern Amazonian landscape (Fig. 1b) by recording forest

status from an aeroplane at 1,104 sample points along 750 km of transects, late in the 1998 dry season. Approximately 9% of the sample points in this study area were recently-burned standing forest, in which ash was visible on the forest floor. The full extent of forest surface fire in 1998 is not known.

Forest impoverishment through logging and surface fire causes a significant release of carbon to the atmosphere that is not included in existing estimates of the Amazonian carbon balance^{1,2}. We made a preliminary estimate of the carbon released from logging by multiplying the area of logging within each harvest intensity class (low, moderate and high, Table 1) by a biomass reduction of 5, 10 and 20%, respectively^{9,10,16}. (We assumed that half of the biomass that is killed or damaged either remains alive or makes its way into long-lived wood products.) In 1996, logging released approximately 4–7% of the net annual carbon release estimated for deforestation in Brazilian Amazonia (about 0.3×10^9 Mg yr⁻¹)¹. The potential for carbon release from forest surface fire, however, is much bigger. For example, if just one-fifth of the forests that had depleted soil water in 1998 (Fig. 1b) had caught fire (killing 20% of forest biomass), net carbon emissions from this region would have more than doubled current estimates^{1,2}, thus rising to a total of 10% of the net annual carbon emissions stemming from human activities worldwide^{1,3}. This fire-mediated release of carbon to the atmosphere adds to the



Figure 2 Forest cover in the vicinity of Paragominas, Pará State. **a**, Landsat TM image, 1991, classified as non-forest (pasture, agriculture, secondary forest) and forest. This type of classification is the basis of the Brazilian government's deforestation estimates⁴. According to this analysis, 62% of this landscape supports forest. **b**, The same Landsat TM image with data from interviews of ranch owners (see text) and areas of selective logging detected in the image¹⁵, showing the area of forest that has been logged, and that which has been logged and burned. According to this analysis, only about a tenth of the area conventionally classified as forest supports forest that has not been disturbed.

carbon release that may be provoked by changes in forest metabolism during ENSO events, as predicted by ecosystem models²⁵.

Logging and fire can virtually eliminate previously undisturbed forest in regions with seasonal drought and high concentrations of wood mills, such as Paragominas in eastern Amazonia (Fig. 1b). Thirty years after settlement, 62% of the land surface in a 3,600-km² area surrounding Paragominas is classified as "forested" using conventional deforestation mapping techniques (Fig. 2a); one-half of this land surface is mandatory forest reserve on private land. But when we map those forests that have been logged or burned (based on our interviews of landholders and detection of logging and forest fire scars in Landsat TM images¹⁵), we find that only about a tenth of the area classified as forest supports undisturbed forest. (Fig. 2b). This 'cryptic' forest impoverishment may be even more common in some other Amazonian regions. Landholders reported a higher incidence of forest surface fires in southern Pará and Mato Grosso, where seasonal drought is more severe than in Paragominas.

Satellite-based deforestation monitoring is an essential tool in studies of human effects on tropical forests because it documents the most extreme form of land use, over large areas, and at low cost. But this monitoring needs to be expanded to include forests affected by logging and surface fire if it is to accurately reflect the full magnitude of human influences on tropical forests. Large-scale burning of tropical forest during severe ENSO episodes may impoverish vast areas of these species- and carbon-rich ecosystems; such episodes are increasing in frequency, possibly in response to the accumulation of greenhouse gases in the atmosphere²⁶. These considerations point to the need to either restrict the logging industry in Amazonia, or to replace conventional logging practices in moist tropical forest regions with low-impact harvest techniques^{17,27}. Cost-effective investments in the prevention of accidental forest fires by Amazonian farmers and ranchers are also needed²⁸. Both of these changes are unlikely to occur unless access to these forest lands provided by expanding road networks, electrical grids and water transport systems is sharply curtailed²⁸. □

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An amniote-like skeleton from the Early Carboniferous of Scotland

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The origin of tetrapods occurred in the Late Devonian period¹, and the earliest known taxa were aquatic². A gap of 30 million years has separated these early forms from the first record of terrestrial tetrapods, in the Late Viséan (Early Carboniferous)³. Here we report the discovery of a small, highly ossified, post-cranial skeleton of a terrestrially adapted, amniote-like tetrapod from the Mid Viséan; this specimen shows the earliest known pentadactyl manus. The skeleton is associated with a gracile humerus that has a constricted shaft and exhibits torsion between proximal and distal articulations. These features are associated with the maintenance of postural support and are strong evidence of locomotion on land⁴. The specimen pushes back the known occurrence of terrestrial vertebrates closer to the origin of tetrapods. Phylogenetic analysis places this new animal close to undisputed amniotes occurring in the Westphalian, indicating that, by the Mid–Late Viséan, amniotes already had a long, but previously unrecorded, history. The origin of amniotes seems to have occurred early in the Carboniferous and was part of a rapid diversification of tetrapods at this time^{3,5,6}.

The specimen occurs in a loose, weathered block of the Cheese Bay Shrimp Bed⁷. The Shrimp Bed is a localized facies within a sequence of discontinuous exposures interrupted by intrusions and faults, bounded by the Garleton Hills Volcanic Rocks and the MacGregor Marine Bands⁸. It is considered to be equivalent in age to the Wardie Shales (Fig. 1), in which the snake-like tetrapod *Lethiscus* was found⁵. The laminated dolomites and mudstones containing the Shrimp Bed were probably deposited in a thermally stratified freshwater lake or brackish lagoon⁷. The shrimp *Tealliocaris* is abundant and specimens show excellent preservation of delicate antennae and legs, indicating little postmortem disturbance⁹. The fauna also includes hydroids, scorpions¹⁰ and occasional specimens of fishes found elsewhere in the Scottish Viséan^{7,11}. The tetrapod described here is the only one to be recorded from this locality.

Tetrapoda Goodrich 1930

Reptiliomorpha Säve-Söderbergh 1934

Undesignated family

Casineria kiddi gen. et sp. nov.

Etymology. caseus (Latin): cheese; sinus (Latin): bay; *kiddi*: in honour of Nicolas Kidd, who discovered the specimen.

Holotype. National Museums of Scotland NMS G1993.54.1, a postcranial skeleton in part and counterpart.

Locality. Cheese Bay, Gullane, East Lothian, Scotland.

Horizon. Cheese Bay Shrimp Bed, Gullane Formation¹².

Age. Asbian, Viséan, Lower Carboniferous.

Diagnosis. Small, well-ossified reptiliomorph (preserved length 150 mm). Approximately 24 gastrocentrous presacral vertebrae; pleurocentrum longer than wedge-shaped intercentrum; low neural spines. Separate scapular blade and coracoid, long, narrow, parallel-sided cleithrum, concave distally; clavicle with symmetrical oval ventral plate and narrow dorsal stem. Gracile, waisted humerus with distinct shaft and evidence of torsion; length of entepicondyle 33% length of humerus; rod-like radius and ulna; carpals unossified; five-digit manus, phalangeal formula 2 ?3 ?4 5 4. Pelvis with long postiliac process. Ventral squamation of ovoid scales.

The specimen, split into part and counterpart halves, consists of

an almost entire articulated postcranial skeleton and the impression of a small part of the lower jaw (Fig. 2). Little detail is visible on most of the original surface but preparation has revealed exquisite preservation of some of the manual phalanges, exposed parts of the humerus, clavicle and cleithrum, and has clarified the vertebral structure. The hindlimbs and pelvic girdle, preserved on the part only, have been extensively weathered and reveal little useful detail.

The vertebral column is preserved in lateral view. The atlas and axis vertebrae have not been identified. The first three cervical segments and a series of five posterior trunk vertebrae are gastrocentrous, with the pleurocentrum being higher and longer than the wedge-shaped intercentrum (Fig. 2). Cervical and trunk neural arches have well-formed zygapophyses. The neural spines are short but increase in anteroposterior length towards the sacrum. All of the presacral vertebrae appear to bear ribs, with the shorter cervicals being slightly more expanded than the curved trunk ribs. Short pointed ribs are associated with at least three presacral vertebrae, and at least one pair of elongate postsacral ribs is present. All visible rib heads have confluent capitulum and tuberculum.

The left pectoral girdle and forelimb are clearly visible (Fig. 3).

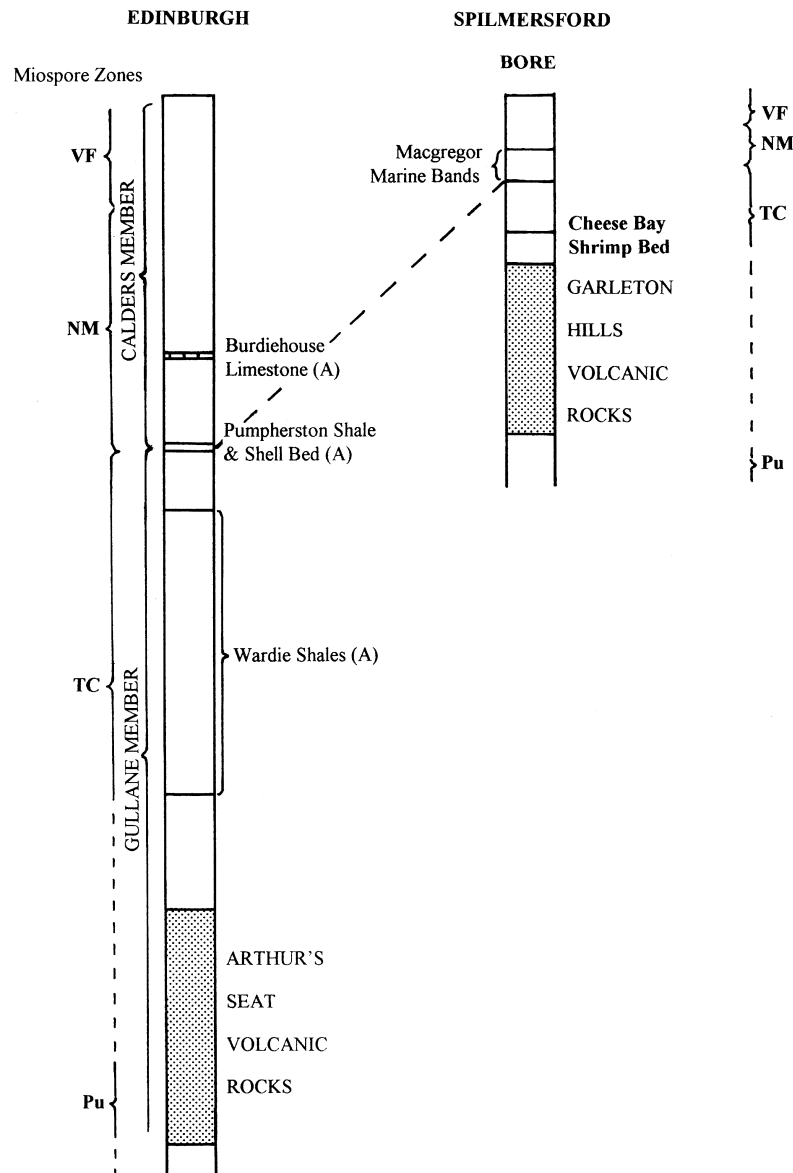


Figure 1 Comparative vertical sections of the Calciferous Sandstone Measures in Edinburgh and East Lothian (Spilmersford), after refs 8, 12. A, tetrapod locality. Miospore zones: VF, *Tripartites vetustus*-*Rotaspora fracta*; NM, *Raistrickia nigra*-*Triquitrites marginatus*; TC, *Perotrilites tessellatus*-*Schulzospira campyloptera*; Pu, *Lycospora pusilla*.

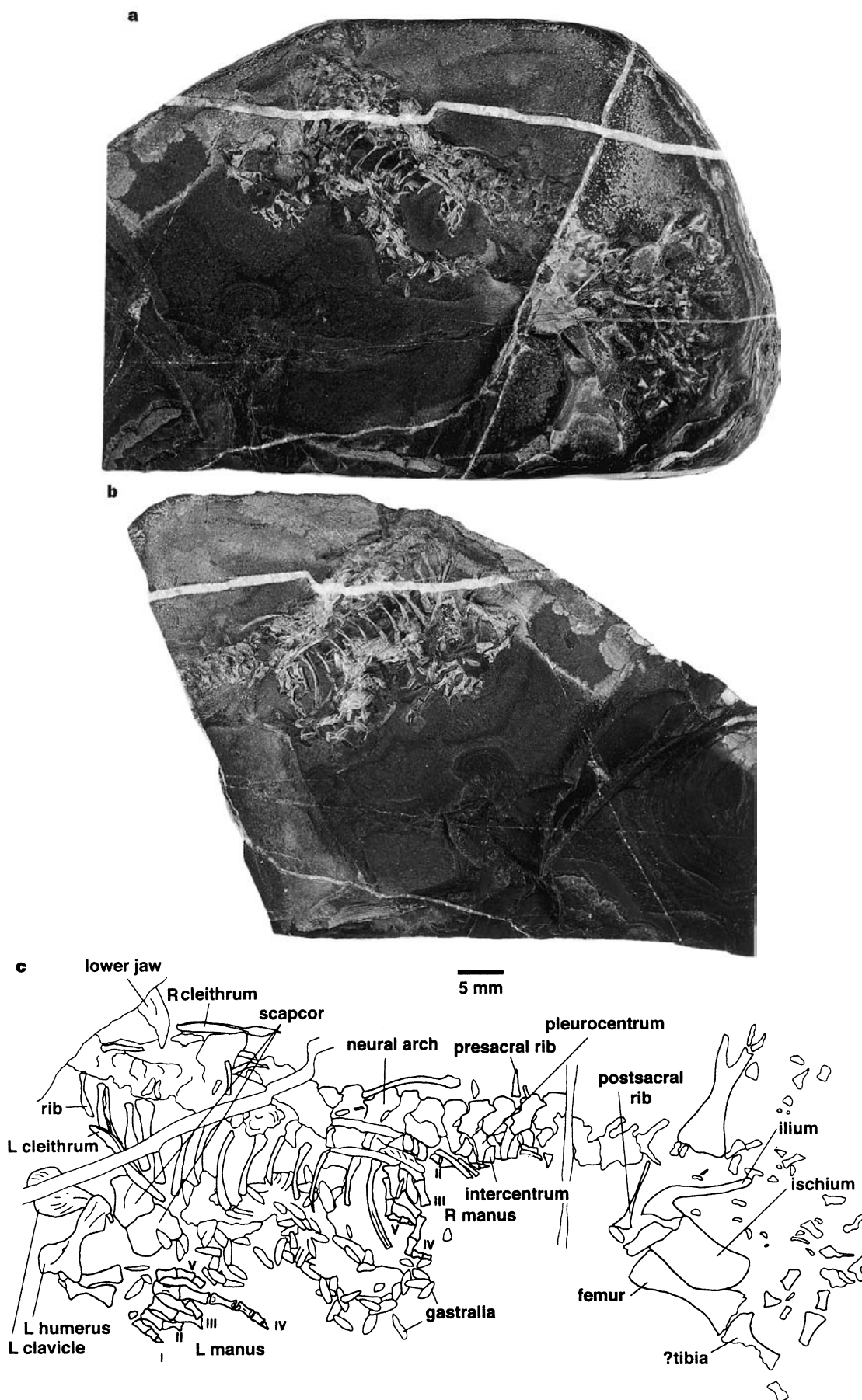


Figure 2 *Casineria kiddi* (NMS G1993.54.1) from the Cheese Bay Shrimp Bed. **a, b**, Part and counterpart halves; shown actual size. **c**, Composite outline drawing.

The poorly ossified endochondral scapulocoracoid shows separate scapular and coracoid portions united at a well-defined suture. This feature is unusual among early tetrapods, and has been reported only in *Whatcheeria deltae* from the Viséan of Iowa, which is possibly a primitive reptiliomorph⁶, and in diadectomorphs,

whereas true amniotes have three centres of ossification¹³. The narrow, parallel-sided cleithra most closely resembles those of the early diapsid *Petrolacosaurus*¹⁴ and the clavicle has an almost symmetrical, oval ventral plate with some internal ridging and a narrow, tapering dorsal stem. The clavicle is broader than in most

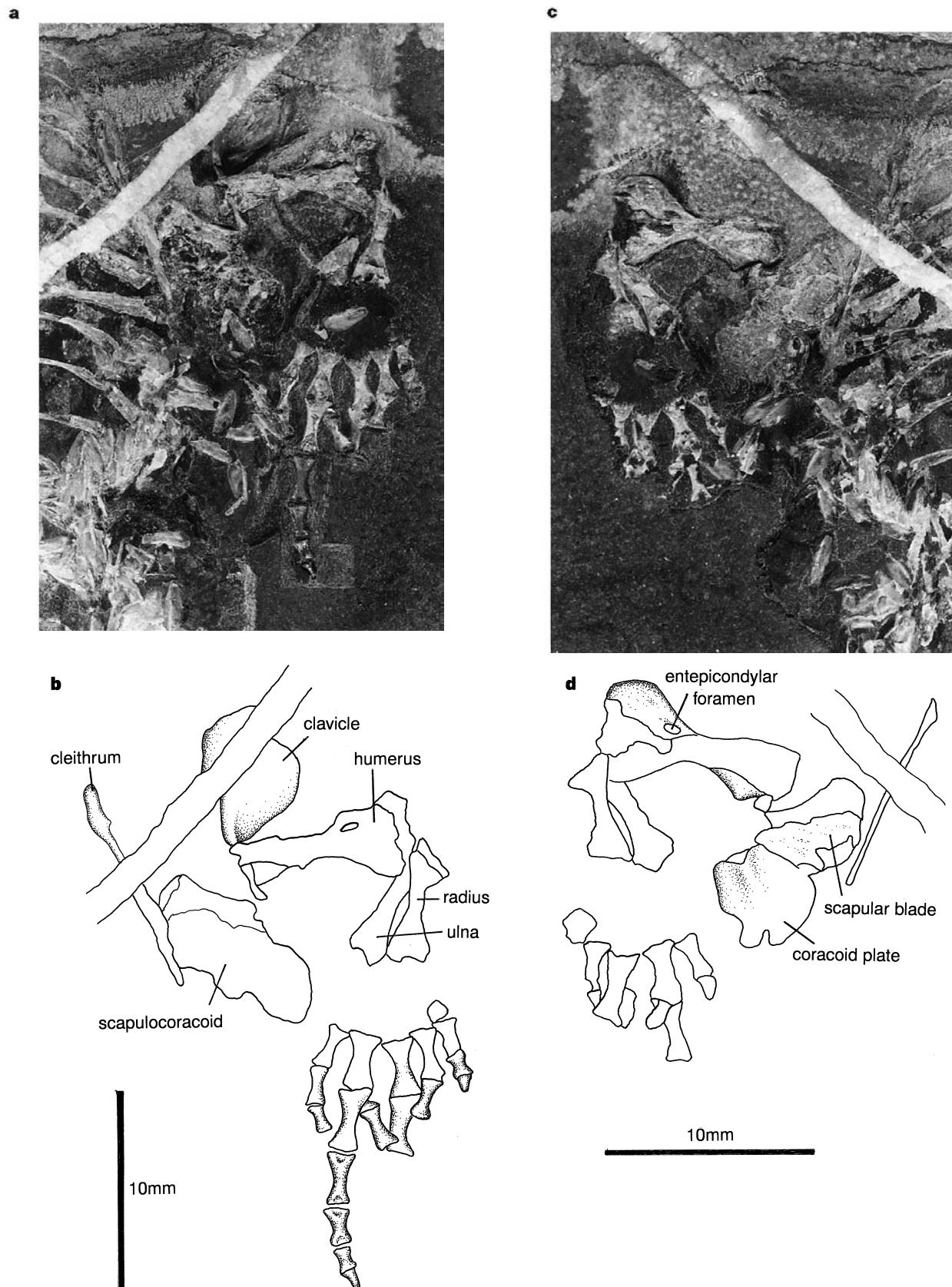


Figure 3 *Casineria kiddi* (NMS G1993.54.1) pectoral girdle and forelimb. **a, b**, Counterpart block. **c, d**, Part block. Undamaged bone surface shaded.

other early amniotes or microsaur.

The overall structure of the humerus is visible but the area around the supinator process is not exposed. The proximal and distal ends are oriented at right angles and separated by a narrow shaft. The degree of twist thus resembles that in the amniotes and *Westlothiana*¹⁵ rather than in other early tetrapods. The length of the entepicondyle is about 33% the length of the humerus, as it is in other amniotes and *Westlothiana*. This contrasts with early tetrapods such as *Acanthostega*¹⁶ and *Eoherpeton*¹⁷, in which the length of the entepicondyle is 50% of that of the humerus. Reduction of the entepicondyle, migration of the muscle attachments proximally and offset of the head from the distal end among early tetrapod humeri transform it from the robust L-shape found in early aquatic forms to the shafted humerus of predominantly terrestrial ones¹⁶.

Both manus are preserved, exposed in ventral view, giving complete phalangeal counts for digits 2, 4 and 5. At least three phalanges are preserved in digit 3, suggesting a complete count of 2 3 4 5 4 as in *Paleothyris*¹⁸ and *Captorhinus*¹⁹. Each end of the phalanges shows a notch in the flexor surface that would have held a substantial ligament to the next phalanx. These are not known in non-amniote tetrapods and indicate the ability to grip, as do the unguals which are curved ventrally into claws as they are in many early amniotes.

One partial pelvic girdle half is preserved, probably the left. The ilium shows an elongate and slender posterior process, but no dorsal blade is preserved. However, the nature of the preservation does not allow us to infer its absence. Conspicuous oval ventral scales have their external surface ornamented with fine striations.

We carried out a phylogenetic analysis to assess the position of *Casineria*. The results are not very robust, but nonetheless appear to place *Casineria* not only on the amniote stem but also among the true amniotes of the Late Carboniferous (Fig. 4). It could thus be an amniote, pre-dating not only the earliest true amniotes from the Westphalian, but also the earliest previously known stem-amniote, *Westlothiana*¹⁵, from East Kirkton.

Casineria is a small tetrapod with an estimated snout–vent length of 85 mm. It is the smallest of all known Lower Carboniferous tetrapods and, within the Carboniferous, only the Westphalian lepospondyl groups include adult taxa that are smaller²⁰. The attainment of small adult size, in which the snout–vent length is no greater than 100 mm, is thought to have been a critical first step

in the evolution of amniotes²¹. *Casineria* provides the first evidence consistent with this hypothesis. Furthermore, it shows the earliest pentadactyl limb, which is clearly terrestrially adapted. All limbs described from earlier animals, that is, those of the Late Devonian, possess more than five digits and belong to arguably aquatic forms¹⁵. The time span between the Devonian forms (about 360 Myr BP) and this Viséan form thus probably represents the maximum period of time over which the pentadactyl limb evolved. The degree of terrestriality exhibited by *Casineria* indicates that the transition to land-dwelling may also have taken place within a period of about 20 million years, and the existence of *Lethiscus* in contemporary deposits attests to a rapid diversification of tetrapods thereafter. The physical resemblance of *Casineria* to known true amniotes from the Late Carboniferous (Westphalian B) and its apparent phylogenetic relationship to these forms indicates that the split between amphibians and amniotes probably also occurred rapidly within this time span. It is clear from the East Kirkton fauna that, by 338 Myr BP, the stem lineages of amphibians, amniotes and several other forms were already well established. □

Methods

An analysis of 21 taxa and 111 characters (78 cranial, 33 postcranial; see Supplementary Information) was carried out using the phylogenetic packages PAUP²² and MacClade²³. Many of the data are derived from a recently published analysis²⁴, but we excluded characters relevant only to fish or Devonian tetrapods, added new postcranial characters, and included several amniotes and two microsauro taxa. We deliberately did not include in the data set established microsauro or amniote apomorphies that could be not be seen in *Casineria*. All characters were run unordered. Source data are included in Supplementary Information.

A heuristic search gave nine most parsimonious trees of length 335 (Fig. 4), with a relatively poor consistency index of 0.47 and a retention index of 0.54. The major variations among these trees occur in the positions of *Whatcheeria* and *Crassigyrinus*, and in the position that *Casineria* occupies among the amniotes. It appears as a sister pairing to *Westlothiana*, *Paleothyris* or *Petrolacosaurus*, remaining within the amniote grouping in all trees. An extra search that included phalanx number on all manual and three pedal digits placed *Casineria* as sister taxon to *Paleothyris* in three most parsimonious trees; however, this placement seems to be supported only by the presence of four phalanges in manual digit 5, and we regard it as unlikely. The consensus of the nine most parsimonious trees places all of the amniote taxa into an unresolved polytomy, united by characters 29 (a process on the premaxilla, convergent with temnospondyls), 50 (absence of ectopterygoid shagreen) and 88 (proportions of the entepicondyle). The microsaur taxa fall out as a group at the next node, supporting their commonly accepted position as stem amniotes. A search for all trees one step longer produced more than 100 equally parsimonious trees and we ended the analysis before completion. As the specimen lacks a head, we attempted an analysis of only the postcranial characters. This analysis produced more than 200 trees, and again was terminated before completion.

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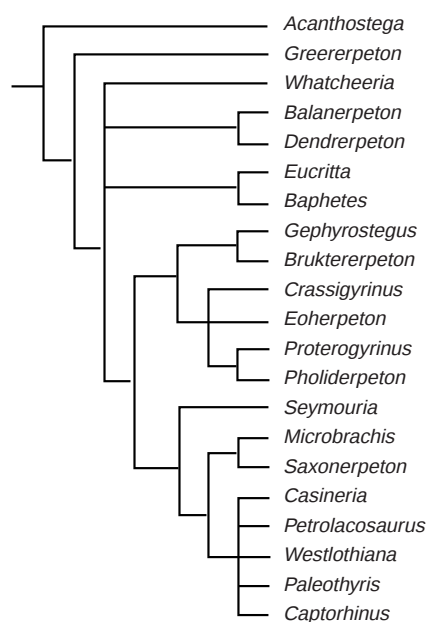


Figure 4 Consensus cladogram drawn from the nine most parsimonious trees of length 335.

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Supplementary information is available on Nature's World-Wide Web site or as paper copy from the London editorial office of Nature.

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Two transmembrane aspartates in presenilin-1 required for presenilin endoproteolysis and γ -secretase activity

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Accumulation of the amyloid- β protein (A β) in the cerebral cortex is an early and invariant event in the pathogenesis of Alzheimer's disease. The final step in the generation of A β from the β -amyloid precursor protein is an apparently intramembranous proteolysis by the elusive γ -secretase(s)¹. The most common cause of familial Alzheimer's disease is mutation of the genes encoding presenilins 1 and 2, which alters γ -secretase activity to increase the production of the highly amyloidogenic A β ₄₂ isoform². Moreover, deletion of presenilin-1 in mice greatly reduces γ -secretase activity³, indicating that presenilin-1 mediates most of this proteolytic event. Here we report that mutation of either of two conserved transmembrane (TM) aspartate residues in presenilin-1, Asp 257 (in TM6) and Asp 385 (in TM7), substantially reduces A β production and increases the amounts of the carboxy-terminal fragments of β -amyloid precursor protein that are the substrates of γ -secretase. We observed these effects in three different cell lines as well as in cell-free microsomes. Either of the Asp $\rightarrow$ Ala mutations also prevented the normal endoproteolysis of presenilin-1 in the TM6 $\rightarrow$ TM7 cytoplasmic loop. In a functional presenilin-1 variant (carrying a deletion in exon 9) that is associated with familial Alzheimer's disease and which does

not require this cleavage⁴, the Asp 385 $\rightarrow$ Ala mutation still inhibited γ -secretase activity. Our results indicate that the two transmembrane aspartate residues are critical for both presenilin-1 endoproteolysis and γ -secretase activity, and suggest that presenilin 1 is either a unique diaspartyl cofactor for γ -secretase or is itself γ -secretase, an autoactivated intramembranous aspartyl protease.

The β -amyloid precursor protein (APP) is cleaved by α - and β -secretases (Fig. 1a), causing the release of soluble derivatives of the protein (α -APPs and β -APPs) and the retention of membrane-anchored 83- and 99-amino-acid C-terminal fragments (C83 and C99)¹. These fragments serve as substrates for γ -secretase, generating the 4K A β fragment from C99 and a 3K peptide, p3, from C83 (ref. 5). A limited portion of presenilin-1 (PS1), which has eight transmembrane segments⁶ (Fig. 1a), undergoes endoproteolysis within the exon-9 region of the cytosolic loop between TM6 and TM7 (more specifically, within the hydrophobic sequence Thr 291–Ala 299)^{7,8}, and the resultant N- and C-terminal fragments form 1:1 heterodimers that have a much longer half-life than the holoprotein and are thought to be the biologically active form of PS1 (ref. 9). The

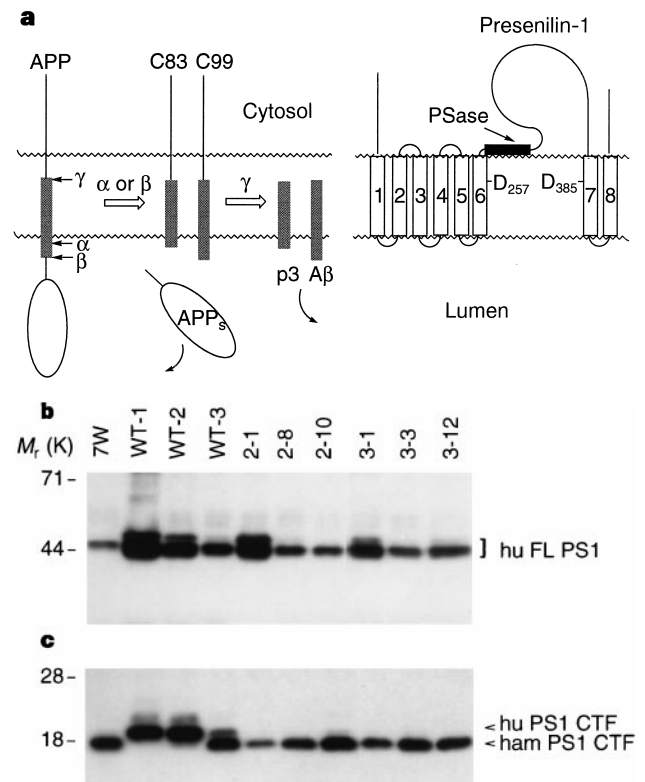


Figure 1 Expression of wild-type and TM Asp $\rightarrow$ Ala mutant PS1 holoproteins and CTFs in stable transfectants. **a**, Structures of APP (left) and PS1 (right), proteolytic processing sites of APP (cleaved by α , β and γ secretases) and PS1 (cleaved by PSase), and locations of Asp257 in TM6 and Asp385 in TM7 of PS1. **b**, PS1 holoproteins (precipitated by antibody 4627 and blotted with 13A11) from CHO cell line 7W stably expressing human APP751 (lane 1), and 7W cell lines stably co-expressing wild-type PS1 (clones WT-1, WT-2 and WT-3 in lanes 2–4), D257A PS1 (clones 2-1, 2-8 and 2-10 in lanes 5–7) or D385A PS1 (clones 3-1, 3-3 and 3-12 in lanes 8–10). Lines with high expression of PS1 characteristically have two holoproteins, both of which have been sequenced and are SDS-stable conformers of full-length (FL) PS1 (ref. 12). **c**, PS1 CTFs immunoprecipitated and blotted as in **b** from the same cell lines. Exogenous human (hu) and endogenous hamster (ham) CTFs are indicated. Replacement of hamster by human CTF is complete in the high-PS1-expressing WT-1 and WT-2 lines, partial⁷ in WT-3, and does not occur in any of the six Asp-mutant lines. In **b**, **c**, the migration positions of relative molecular mass markers are indicated on the left.

specific function of the presenilins, including their role in A β generation, is unknown. The aspartate residues in TM6 and TM7 are the only two potentially charged residues clearly within the theoretical membrane boundaries of PS1 and PS2 (ref. 2). Where aspartates are found within TM regions of other proteins (for example, adrenergic receptors¹⁰), they can be critical for function. Thus, the conserved TM aspartates in presenilins may be required for their functions, including their ability to mediate γ -secretase activity.

We constructed plasmids containing wild-type, D257A or D385A human PS1 complementary DNA, and stably transfected Chinese hamster ovary (CHO) cells overexpressing the 751-residue wild-type human APP (APP751) with each PS1 plasmid. The processing of human APP and PS1 in CHO cells has been extensively characterized^{11,12}. Using antibody 4627 for immunoprecipitation from cell lysates and antibody 13A11 for western blotting (both raised against the PS1 C-terminal fragment (CTF)), we found that expression of the ~44K wild-type and mutant PS1 holoproteins varied between nine independent stable cell lines (Fig. 1b). Pulse-chase experiments done as controls revealed no difference in the overall metabolic turnover of the mutant as compared to the wild-type holoproteins (both have short half-lives) (results not shown), and membrane fractionation revealed that the subcellular distribution of the holoproteins was normal (see below), indicating that the single Asp $\rightarrow$ Ala substitutions did not detectably alter the mem-

brane insertion and transport of the holoproteins. Furthermore, the Asp $\rightarrow$ Ala substitutions did not appear to increase PS1 aggregation, as no high-molecular-weight SDS-stable bands were seen (results not shown).

Consistent with previous evidence that PS1 N- and C-terminal fragments are tightly regulated by competition for limiting cellular factors¹³, overexpression of wild-type human PS1 led to the replacement of endogenous hamster CTFs by the human CTFs (Fig. 1c). The extent of replacement depended on the amount of wild-type human PS1 expressed (Fig. 1b, c; lanes 2–4), as reported⁷. In contrast, there was no endoproteolysis of D257A or D385A PS1 to human CTFs (lanes 5–10). Apparently, mutation of either of the two PS1 transmembrane aspartates prevented endoproteolysis in the TM6 $\rightarrow$ TM7 loop. Endogenous hamster CTFs were not replaced in most of the cell lines expressing mutant human PS1; an exception was the D257A cell line 2-1, the highest expressor of mutant holoprotein (Fig. 1b, lane 5), in which endogenous CTFs were markedly decreased (Fig. 1c, lane 5); the second-highest expressor, the mutant line 3-1, also showed a decrease in endogenous CTFs (Fig. 1c, lane 8).

Cells transfected with wild-type or Asp $\rightarrow$ Ala mutant PS1 showed comparable expression of the 110K–120K N-, and N and O-glycosylated APP holoproteins (Fig. 2a). Likewise, the amounts of the soluble ectodomain derivatives generated by the β - and α -secretases (β -APP_s and α -APP_s) were essentially unchanged (Fig.

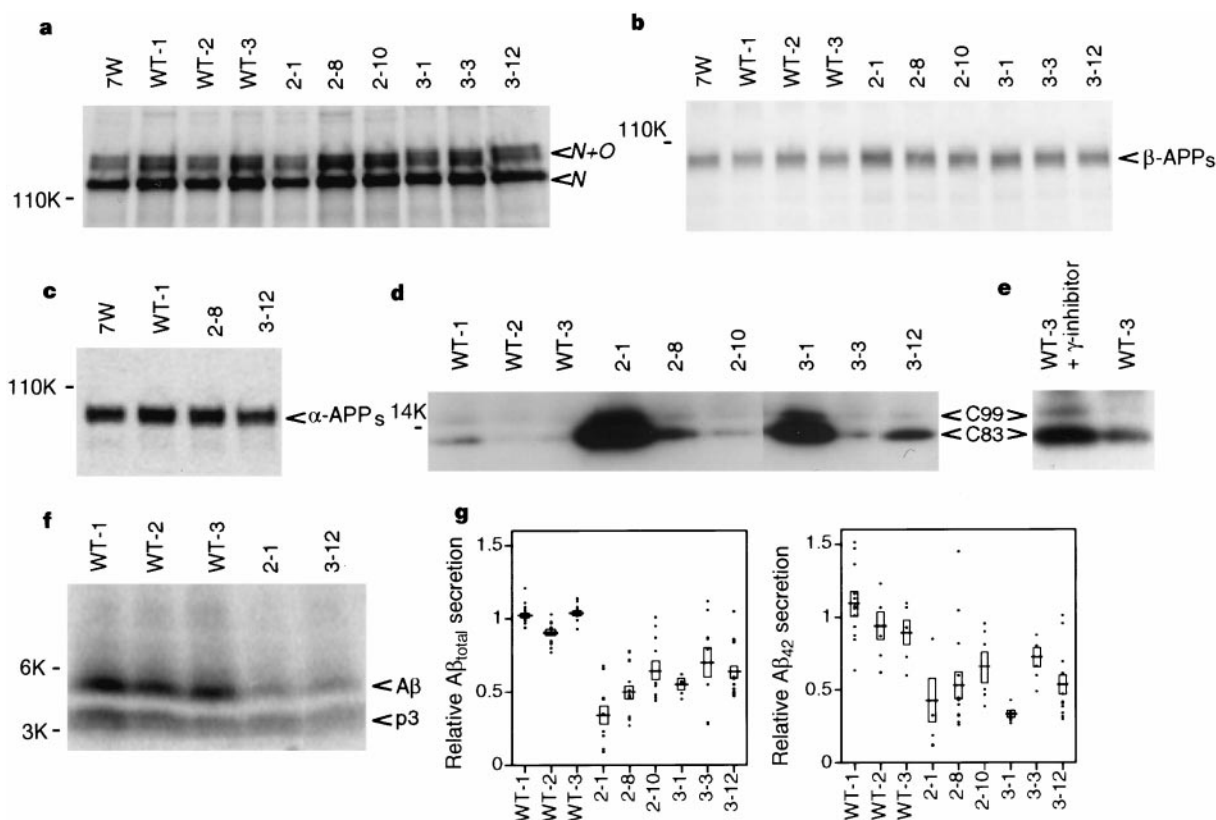


Figure 2 Effect of wild-type and transmembrane Asp $\rightarrow$ Ala mutant PS1 on APP processing in stable CHO transfectants. **a**, N- and N+O-glycosylated holoAPP metabolically labelled with [³⁵S]Met and precipitated from lysates with antibody C7. Cell lines were as described for Fig. 1b. **b**, β -APP_s precipitated from medium with antibody 192. **c**, α -APP_s precipitated from medium with antibody 1736. The other six lines gave similar results. **d**, C83 and C99 precipitated with antibody C7 were blotted with 13G8; all lines have C83 and C99, although C99 is barely visible in WT-2. C83 and C99 levels from 7W cells (not shown) were comparable to those for WT cell lines. **e**, Line WT-3 was left untreated (right) or treated with a published

γ -secretase inhibitor¹⁴ for 4 h at 25 μ M (left), and the lysates were precipitated and blotted as for **d**. **f**, A β and p3 precipitated from medium of metabolically labelled cells by antibody 1280 against A β . The decreases in A β in lines 2-1 and 3-12 as shown by phosphorimaging were similar to those quantified by ELISA (**g**); the amount of p3 in these lines was reduced by ~40–50%. **g**, ELISAs for total A β and A β ₄₂. Relative amounts of A β (dots) were determined by calculating the ratio of the amount of A β secreted by each mutant cell line to the mean amount of A β secreted by the 3 WT lines in each experiment (normalized to 1). Boxes, s.e.m.; horizontal bars, mean.

2b, c). In contrast, expression of D257A or D385A PS1 resulted in consistently increased amounts of C99 and C83 compared to wild-type PS1 (Fig. 2d). The increase correlated roughly with the level of expression of mutant holoprotein (Fig. 1b); for example, the PS1 D257A-containing cell line 2-1, which expressed the most mutant holoprotein and markedly reduced levels of endogenous PS1 C-terminal fragments (Fig. 1b and c, lane 5), always produced the most C99/C83 (Fig. 2d, lane 4), and cell line 3-1 produced the next highest levels of mutant PS1 and C99/C83 (Fig. 2d, lane 7). The effects of the PS1 mutations on the amounts of C99/C83 produced were similar to those caused by a peptidomimetic γ -secretase inhibitor (Fig. 2e)¹⁴. Thus, accumulation of C99 and C83 caused by the Asp $\rightarrow$ Ala mutations results from interference with γ -secretase activity and not from increased α - and β -secretase cleavage of holoAPP, consistent with the results of deleting the PS1 gene³.

All of the cell lines stably expressing Asp $\rightarrow$ Ala mutant PS1 secreted substantially less total A β (mean $57 \pm 3\%$) and A β ₄₂ ($53 \pm 4\%$) than cell lines expressing wild-type PS1 ($P < 0.001$ in each mutant line), as assessed by sandwich ELISA (Fig. 2g). This decrease in A β secretion in the mutant PS1-containing cell lines was confirmed by [³⁵S]-methionine labelling and immunoprecipitation of medium with A β antibody 1280 (Fig. 2f). In general, the decrease in A β secretion among the six stable mutant lines correlated roughly with the amount of expression of the mutant holoprotein, with cell line 2-1 showing the greatest inhibition of total A β production ($36 \pm 9\%$ ($n = 10$) of the mean A β production of the three wild-type PS1 cell lines), a reduction approaching that obtained by deleting the PS1 gene³. We assume that the residual A β production is due to PS2 (ref. 3). Moreover, the levels of total A β (principally A β ₄₀) and A β ₄₂ both fell synchronously as the expression of the Asp $\rightarrow$ Ala mutant holoproteins rose (compare Figs 2g and 1b), again mimicking the PS1-knockout effect. Thus, these single aspartate point mutations have the same effect on APP processing (inhibiting γ -secretase activity) as deleting the entire PS1 gene and appear to act as dominant-negative effectors with respect to endogenous PS1. No other PS1 mutation reported to date has these effects, underscoring the critical importance of each of the two TM aspartates for PS1 function.

We assessed the effect of the PS1 Asp $\rightarrow$ Ala mutations directly on C99 processing by transiently transfecting monkey COS-1 cells with plasmid pCI-C99, encoding C99 fused to the APP signal sequence. Cells transfected with C99 alone contained large amounts of this protein compared with COS-1 cells transfected with APP alone, in which C99 was not detectable (not shown). Co-transfection of C99 with D257A or D385A PS1 substantially increased C99 compared with wild-type PS1 (Fig. 3a), again indicating that elimination of either aspartate protects C99 against catabolism by γ -secretase. Interestingly, blocking γ -secretase activity resulted in accumulation of not only C99 but also of the endogenous C83 substrate of γ -

secretase (Fig. 3a). Transfection of either Asp $\rightarrow$ Ala-mutant PS1 alone (without C99) also gave increased amounts of C83 compared with wild-type PS1 (not shown), indicating that the PS1 mutants block the normal catabolism of endogenous monkey kidney C83. To examine a third cell type, we also transiently transfected the PS1 Asp $\rightarrow$ Ala mutants into a human kidney 293 cell line stably expressing the K595N/M596L ('Swedish') double mutation of human APP¹. Again, both D257A and D385A PS1 caused markedly increased C99 and C83 compared with wild-type PS1 (Fig. 3b, lanes 1–4). Thus, the PS1 Asp $\rightarrow$ Ala mutants interfered with γ -secretase processing of C99 and C83 in cell lines from three different species (including human), and the effect was observed for endogenous and exogenous γ -secretase substrates and in transient as well as multiple stable transfectants. A β production in these transiently transfected cells was too variable and too close to the limit of ELISA detection for quantification.

The above results indicate that the two PS1 transmembrane aspartates are essential for PS1 endoproteolysis and γ -secretase activity. However, it was unclear whether the reduced γ -secretase activity resulted solely from eliminating endoproteolysis of the mutant PS1 holoproteins. We therefore introduced the D385A mutation into PS1 Δ E9, a functional PS1 variant that lacks exon 9 and does not undergo conventional endoproteolysis^{4,7}, and transfected the 293 cells expressing APP695 K595N/M596L with this PS1 variant. As expected, the mutant holoproteins were expressed, but PS1 C-terminal fragments were not generated from either PS1 Δ E9 or its D385A counterpart (not shown). Expression of PS1 Δ E9, a mutation that causes familial Alzheimer's disease, did not alter either C83 or C99 levels (Fig. 3b, lane 5) compared to vector or wild-type PS1 (lanes 1 and 2). However, cells transfected with Δ E9-D385A PS1 accumulated substantially more C83 and C99 than those transfected with PS1 Δ E9 (lanes 5 and 6). Thus, this transmembrane aspartate is independently critical for γ -secretase activity even in a PS1 variant that does not require endoproteolysis to function in APP processing.

Because a critical negative charge was lost in each of the Asp $\rightarrow$ Ala mutations, it was possible that the protein was misfolded (that is, the aspartates serve an important structural, rather than functional, role). We therefore introduced a glutamate mutation into PS1 to conserve the charge (D257E) and transfected this construct into CHO 7W cells expressing APP751 and into HEK 293 cells expressing APP695 K595N/M596L. In each cell line, expression of D257E-mutant PS1 increased C83 and C99 compared with wild-type PS1 (Fig. 3c; only shown for 7W cells), demonstrating that aspartate is specifically required in this position.

We examined the ability of microsomes isolated from selected cell lines to generate A β from C99 *in vitro*. Microsomes were prepared from the CHO cells stably expressing wild-type or Asp $\rightarrow$ Ala mutant PS1. Immunoprecipitation and western blotting with anti-

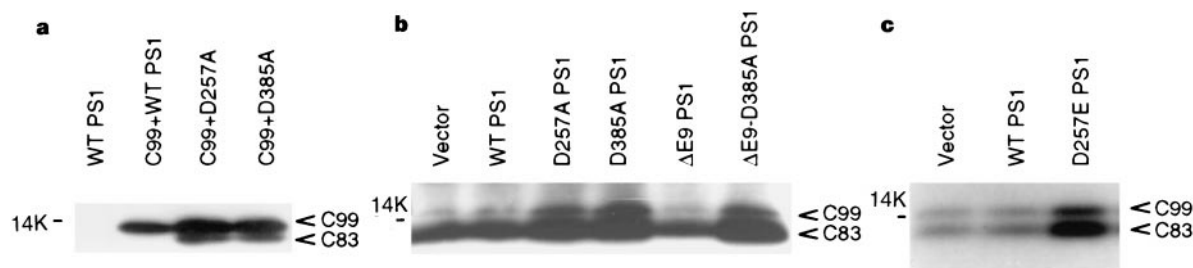


Figure 3 Effect of wild-type and transmembrane Asp $\rightarrow$ Ala mutant PS1 on APP processing in transient transfectants. **a**, C83 and C99 (precipitated and blotted as for Fig. 2d) from monkey COS-1 cells co-transfected with C99 and either WT or mutant PS1. Note the strong C83 band when Asp-mutant PS1 is expressed. **b**, C99 and C83 from human 293 cells stably transfected with human APP695 K595N/

M596L and transiently transfected with vector alone, WT PS1, D257A PS1, D385A PS1, Δ E9 PS1 or Δ E9-D385A PS1. **c**, C99 and C83 from CHO cell line 7W stably transfected with human APP751 and transiently transfected with vector alone, WT PS1, or Asp $\rightarrow$ Glu (D257E) PS1.

body 4627 showed that the PS1 holoproteins were present in each microsomal preparation but, as observed in whole-cell lysates, microsomes isolated from the cells expressing Asp → Ala mutant PS1 did not have the corresponding CTFs (not shown). The microsomes were added to a cell-free rabbit reticulocyte transcription/translation system containing [³⁵S]Met and pCI-C99, a DNA construct with a T7 promoter suitable for C99 expression in this system. After sufficient time at 30 °C for C99 expression, samples were incubated at 37 °C for 1 h under either neutral or mildly acidic (pH 6.4) conditions. The latter condition was chosen because γ -secretase appears to have some properties of an aspartyl protease¹⁵. Reactions were quenched, precleared, and immunoprecipitated with A β antibody 1280. When the incubation was performed at neutral pH, C99 was well expressed, but virtually no A β was detected (Fig. 4a). However, under mildly acidic conditions, a new 4K band which co-migrated with ¹²⁵I-labelled A β appeared only with the microsomes purified from cell lines expressing wild-type PS1 (Fig. 4a). Preabsorption of antibody 1280 with A β peptide eliminated the 4K band (not shown).

We also looked for *de novo* A β generation from endogenous, full-length APP in microsomes purified from the same cells stably expressing wild-type PS1 (line WT-1) or Asp → Ala mutant PS1 (lines 2-1 and 3-1). The microsomes were incubated for 4 h at 37 °C (or at -80 °C to determine basal A β levels), and their contents were then assayed by A β ELISA. Microsomes expressing wild-type PS1 produced a 2–3 fold increase (mean: 160 pg ml⁻¹; *n* = 3, *P* < 0.05) in total A β over basal levels (mean: 60 pg ml⁻¹; *n* = 3), whereas microsomes expressing Asp → Ala mutant PS1 had undetectable basal A β and produced no new A β after incubation at 37 °C. Quantitatively similar results were obtained using an A β ELISA that specifically detects only A β species ending at residue 40,

confirming the occurrence of bona fide γ (40)-secretase cleavage of holoAPP in the PS1 wild-type microsomes. These results in isolated microsomes, both with *in vitro*-generated C99 and intrinsic APP, mirror the inhibition of A β production observed in our Asp → Ala mutant cells (Fig. 2f, g) and in neurons of PS1-knockout mice³, and suggest that PS1 plays a direct role in γ -secretase cleavage, rather than influencing the transport of the component proteins of this reaction. In support of this conclusion, the subcellular distribution of holoAPP, C83 and C99 after gradient fractionation of total microsomes was the same in the presence (+/+) as in the absence (-/-) of PS1 in mouse fibroblasts¹⁶, and in the presence of wild-type or Asp → Ala mutant PS1 in CHO cells (not shown). Furthermore, aspartate-mutant PS1 showed the same subcellular distribution as wild-type PS1 in CHO cells stably expressing these proteins (Fig. 4b), providing further evidence against a principal role in protein transport for PS1. There were also no changes in the subcellular distribution of endogenous PS1 C-terminal fragments in cells expressing Asp → Ala mutant PS1 compared with wild-type PS1 (not shown), although the amounts of these endogenous fragments in cells expressing Asp → Ala mutant PS1 were reduced compared to wild-type PS1, as expected (Fig. 1c).

It is remarkable that two aspartates located in the middle of two adjacent transmembrane domains are independently critical for two separate proteolytic events: the cleavage of PS1 by the unidentified presenilinase and the cleavage of APP C-terminal fragments C99 and C83 by the unidentified γ -secretase. These aspartates are conserved even in *Caenorhabditis elegans* homologues SEL-12, SPE-4 and HOP-1^{17,18}, indicating that they are functionally important. In support of this idea, the mutation of Asp 385 to Asn eliminates the ability of human PS1 to rescue a lethal egg-laying defect in a *SEL-12* mutant of *C. elegans*¹⁹. Although most of the more than 50 PS mutations associated with familial Alzheimer's disease lie within transmembrane domains², and some of these sites are close to the two aspartate residues, natural mutation of the aspartates themselves has not been found. Such mutations would probably be lethal during embryonic development, as occurs in PS1-knockout mice^{20,21} and should not lead to Alzheimer's disease as they cause a marked decrease in production of both A β ₄₀ and A β ₄₂.

What is the role of presenilins, and of these two transmembrane aspartates in particular, in γ -secretase function? Although the presenilins are mostly found in the endoplasmic reticulum and early Golgi^{16,22}, they are unlikely to be important for protein transport or chaperoning, considering our results with isolated cell-free microsomes and our subcellular fractionation studies. The presenilin aspartates are probably not critical for the translocation of nascent proteins (including APP) as α - and β -secretase processing are normal both in PS1 knock-out mice³ and in cells expressing Asp → Ala mutant PS1 (Fig. 2). The presenilins may be cofactors for γ -secretase, analogous to the sterol-cleavage activating protein, also a multiple-spanning transmembrane protein found in the endoplasmic reticulum, which regulates the site-1 protease that cleaves the sterol regulatory-element-binding protein²³. Although there is no precedence for two transmembrane aspartates being essential for a protease regulator, such a role for the presenilins in γ -secretase activity cannot be excluded.

Alternatively, presenilins themselves could be γ -secretases, which have some of the properties of aspartyl proteases¹⁵. The two transmembrane aspartates are predicted to align with each other (that is, they could associate) and also with the site in the transmembrane domain of APP that is cleaved by γ -secretase (so they could catalyse an intramembrane proteolysis). Our results are consistent with PS1 being an autoactivated aspartyl protease with γ -secretase activity: mutation of either transmembrane aspartate prevents both activities. As substitution of one of these aspartates with glutamate also disrupts γ -secretase activity, the presence of aspartate (not simply its charge) is critical, strongly favouring the

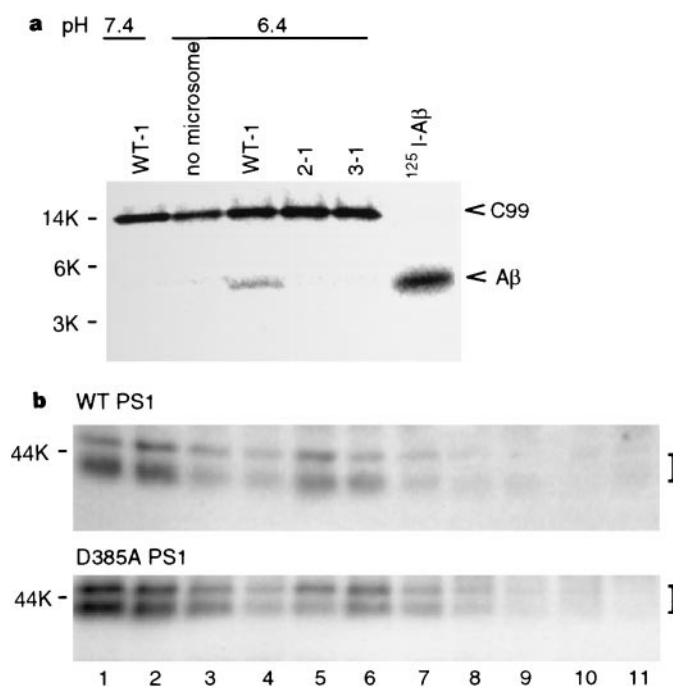


Figure 4 Cell-free generation of A β from isolated microsomes, and subcellular distribution of PS1. **a**, A β precipitated with antibody 1280 from a rabbit reticulocyte *in vitro* transcription/translation system containing [³⁵S]Met, plasmid pCI-C99 and microsomes isolated from the indicated CHO lines stably expressing WT or Asp → Ala mutant PS1. After protein synthesis, microsome reactions were incubated at 37 °C for 1 h under neutral (pH 7.4) or mildly acidic (pH 6.4) conditions (see Methods). **b**, Subcellular fractionation of microsomes isolated from stable cell lines WT-1 and 3-1 through an iodixanol step gradient²⁰. The distribution of the PS1 holoproteins across the 12 fractions is shown by immunoprecipitation and western blotting; no retention or redistribution of the Asp-mutant PS1 was seen.

hypothesis that the presenilins are aspartyl proteases themselves and not regulators. Furthermore, A β is generated in cell-free microsomes containing wild-type PS1 under mildly acidic conditions; aspartyl proteases require two (and only two) activate-site aspartates and function optimally at acidic pH. The fact that overexpression of wild-type presenilins does not increase A β is not inconsistent with the idea that presenilins are γ -secretases, because even overexpressed presenilin is processed to tightly limited amounts of stable amino- and carboxy-terminal fragments, which are probably the active forms of the protein^{7-9,13}. On the other hand, presenilin has no amino-acid sequence homology with known (cytosolic) proteases.

If γ -secretases effect intramembrane proteolysis, these enzymes should have several transmembrane regions, as do PS1 and 2, containing residues involved in catalysis and allowing hydrolytic water to enter through an opening formed by association of the TM domains. There is no clear precedent for a protease with a transmembrane active site, although there are several known multi-spanning transmembrane proteases in the endoplasmic reticulum, including the site-2 protease for the sterol regulatory element-binding protein²⁴ and the yeast Rce1 and Ste24 proteases²⁵ which process prenylated proteins with CAAX carboxy termini. Confirmation that presenilins are γ -secretases will require A β to be generated by artificial membranes expressing only PS and C99. However, this may be difficult if unknown limiting cellular factor(s) are required for the presenilins to function¹³. But even if the two aspartates function as part of a non-proteolytic co-factor in the presenilinase and γ -secretase cleavages, this will have implications for both the biology of proteases and the treatment of Alzheimer's disease and related disorders involving A β accumulation.

Our findings are also important for understanding the role of presenilins in Notch-directed cell-fate decisions during embryonic development. PS1 is part of the signalling pathway from Notch^{21,26}, a receptor that, upon ligand-induced activation, undergoes proteolytic processing which is strikingly similar to that of APP, including cleavage of a C-terminal fragment of Notch within its putative transmembrane region²⁷. PS1 mediates this proteolysis of Notch, which is blocked by several γ -secretase inhibitors²⁸. These findings, together with ours, make PS1 a prime candidate for the γ -secretase-like protease that processes Notch. □

Methods

Plasmids and transfections. Full-length wild-type (WT) or Δ E9 mutant PS1 was used as a template to introduce D257A, D385A or D257E by site-directed mutagenesis, and the genes were inserted into vector pCDNA3.1, which contains a zeocin-resistance gene. The identities of the mutant genes were confirmed by DNA sequencing. For transient expression, plasmids (10 μ g) were transfected into the cell lines in 10-cm dishes using Lipofectamine. 72 h after transfection, cells were either first labelled with [³⁵S]Met for 1 h or collected and then lysed in buffer containing 50 mM Tris, pH 7.6, 1% NP-40, 2 mg ml⁻¹ BSA, 150 mM NaCl, 2 mM EDTA and protease inhibitors. CHO cell lines stably co-expressing APP and mutant PS1 were generated from cell line 7W (ref. 11) (stably transfected with human APP751 and a neomycin-resistance gene) using zeocin and G418 for selection.

Immunoprecipitation, immunoblotting and sandwich ELISA. The PS1, APP, APP₆₉₅ and A β antibodies have all been characterized^{25,28}. Cell lysates adjusted to equal protein concentration were precleared with protein A-Sepharose at 4 °C for 30 min and precipitated with primary antibody and protein A-Sepharose at 4 °C for 2 h. Beads were washed twice with 0.2% NP-40 buffer and heated for 5 min at 65 °C in reducing SDS-sample buffer. The precipitated proteins were resolved by SDS-PAGE on 14, 8–16 or 4–20% gels. Polyvinylidene difluoride immunoblots were developed with peroxidase-conjugated secondary antibody and enhanced chemiluminescence (ECL, Amersham). Immunoprecipitates of proteins labelled with [³⁵S]Met were analysed by phosphorimaging. ELISAs for total A β and A β ₄₂ were performed as described²⁹. Capture antibodies were 266 (to residues 13–28) for total A β and 21F12 (to residues 33–42) for A β ₄₂. Reporter antibody was biotinylated 3D6 (to residues 1–5) in each assay.

In vitro A β generation and subcellular fractionation. Cell homogenates prepared as described¹⁶ were centrifuged at 3,000g, and the supernatant was spun at 100,000g. The microsomal pellet was taken up in a rabbit reticulocyte *in vitro* transcription/translation system suitable for transcription of plasmids containing the T7 promoter (Promega), together with [³⁵S]Met, and the volume was adjusted based on the protein content of the 3,000g supernatant. After addition of plasmid pCI-C99, samples were incubated at 30 °C for 1.5 h to allow transcription and translation to occur, then incubated at 37 °C for 1 h with or without an equal volume of 100 mM sodium citrate buffer, pH 5.6 (final pH, 6.4), quenched with Tris buffer containing 1% NP-40, precleared, and immunoprecipitated by A β antibody 1280. Precipitated proteins were separated by SDS-PAGE on 10–20% gels and analysed by phosphorimaging. Subcellular fractionation was achieved by passing resuspended microsomal pellets through an iodixanol step gradient as described³⁰.

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A presenilin-1-dependent γ -secretase-like protease mediates release of Notch intracellular domain

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Signalling through the receptor protein Notch, which is involved in crucial cell-fate decisions during development, requires ligand-induced cleavage of Notch. This cleavage occurs within the predicted transmembrane domain, releasing the Notch intracellular domain (NICD), and is reminiscent of γ -secretase-mediated cleavage of β -amyloid precursor protein (APP), a critical event in the pathogenesis of Alzheimer's disease. A deficiency in presenilin-1 (PS1) inhibits processing of APP by γ -secretase in mammalian cells, and genetic interactions between Notch and PS1 homologues in *Caenorhabditis elegans* indicate that the presenilins may modulate the Notch signalling pathway¹⁻⁴. Here we report that, in mammalian cells, PS1 deficiency also reduces the proteolytic release of NICD from a truncated Notch construct, thus identifying the specific biochemical step of the Notch signalling pathway that is affected by PS1. Moreover, several γ -secretase inhibitors block this same step in Notch processing, indicating that related protease activities are responsible for cleavage within the predicted transmembrane domains of Notch and APP. Thus the targeting of γ -secretase for the treatment of Alzheimer's disease may risk toxicity caused by reduced Notch signalling.

Notch-1 is synthesized as a type I integral-membrane protein of relative molecular mass 300,000 (M_r 300K) (Fig. 1) that is cleaved by a furin-like protease in the Golgi during trafficking of Notch to the cell surface. The two proteolytic fragments remain associated to form the functional receptor^{5,6}. Following ligand binding, Notch-1 undergoes further cleavage close to or within its transmembrane domain, releasing the NICD. The NICD translocates to the nucleus and modifies transcription of target genes through its association with CSL proteins (where CSL stands for CBF1, Su(H), Lag-1)⁷⁻⁹.

APP is also a type I integral-membrane protein. APP is the precursor of the amyloid- β peptide, which is deposited in the brains of Alzheimer's disease patients. Amyloid- β peptide is derived from APP as a result of cleavage of APP by β - and γ -secretase, whose molecular nature remains unclear. β -Secretase cleaves the extracellular domain of APP, producing a soluble ectodomain and a membrane-associated carboxy-terminal fragment (Fig. 1). γ -Secretase catalyses an intramembranous cleavage of this membrane-associated fragment, resulting in generation of amyloid- β peptide and the production of a C-terminal fragment of APP. Cleavage of APP by α -secretase precludes production of amyloid- β peptide (Fig. 1). The

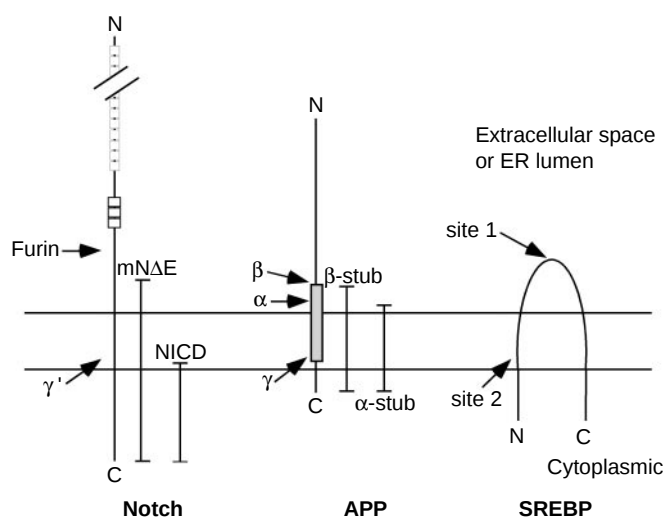


Figure 1 Proteolytic cleavage of Notch, APP and SREBP (not to scale). The proteolytic fragments (NICD and α - and β -stubs) and constructs (mNAE) used to analyse Notch-1 and APP metabolism are indicated, as are the proteases involved in processing (furin, γ -, α -, β - and γ -secretases, and site-1 and site-2 proteases). For a more extensive discussion on the analogies between APP, Notch-1 and SREBP processing, see ref. 30.

functional role(s) of these APP fragments is unknown. Genetic and biochemical studies have implicated the proteolytic processing events that lead to amyloid- β -peptide generation in the pathogenesis of Alzheimer's disease. Mutations in APP and the presenilins (PS1 and PS2) cause familial early-onset Alzheimer's disease, and presenilin mutations lead to increased processing of APP by γ -secretase *in vitro* and *in vivo*¹⁰. PS1-deficient mice show decreased γ -secretase processing of APP⁴ and developmental abnormalities consistent with altered Notch signalling^{2,3}. Genetic interactions between the *notch* homologues *glp-1* and *lin-12* and the *presenilin* homologues *sel-12* and *hop-1* in *C. elegans* provide indirect evidence for the involvement of the presenilins in the Notch signalling pathway^{1,11}. Here we identify the specific step in Notch signalling that is controlled by PS1.

We first investigated whether the absence of PS1 interferes with the normal expression of Notch, as has been suggested². We used brain extracts from PS1-deficient (PS1^{-/-}) mouse embryos at embryonic day (E) 14. As expected, no expression of the amino-terminal or C-terminal fragments of PS1 was found (Fig. 2a and ref. 4). Moreover, in agreement with our previous results obtained from study of neuronal cultures⁴, we observed an accumulation of endogenous mouse APP C-terminal fragments in the same brain extracts, indicating deficient γ -secretase processing of APP when PS1 is absent (Fig. 2a, lower arrow). These fragments are predominantly 'stubs' produced by α -secretase, because endogenous mouse APP is not a good substrate for β -secretase (owing to the presence of amino-acid substitutions between the mouse and the human sequences at the β -secretase site)^{4,12}. In the same homogenates, we detected normal steady-state levels of furin-processed Notch-1 C-terminal fragments, indicating that PS1 is not required for Notch biosynthesis or cleavage by furin within the Golgi (Fig. 2b). PS1-independent Notch maturation was also observed *in vitro*, in mouse fibroblasts derived from PS1^{-/-} embryos (Fig. 2c). We conclude that PS1^{-/-} mouse cells produce normal amounts of mature (furin-cleaved) Notch 1 protein.

The similarities between the proteolytic processing of Notch and APP are striking, especially the final proteolytic events that release the cytoplasmic domains, raising the possibility that PS1 might mediate the intramembranous proteolytic cleavage of Notch-1 to

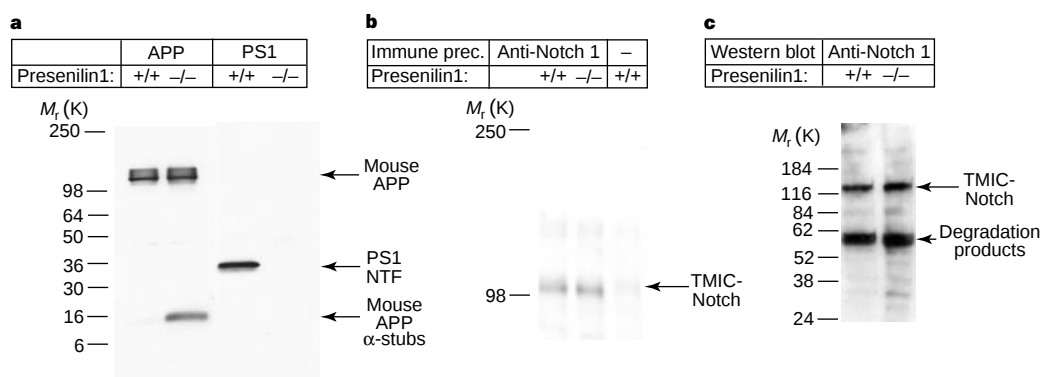


Figure 2 Processing of APP and Notch-1 in PS1^{-/-} mouse brain and MEFs. Proteins were detected in western blots. **a**, Brain-membrane extracts (4–20% SDS-PAGE, 50 μ g per lane) were reacted with APP675–695, an antiserum specific for the APP C terminus, or with an antiserum specific to the PS1 N terminus (PS1NTF). Arrows identify PS1NTF and a mouse APP fragment generated by α -secretase-mediated processing (mouse APP α -stubs). **b**, Notch-1 was

immunoprecipitated from 100 μ g brain extract using IC-antiserum, an anti-Notch-1 antiserum⁵ and stained with the monoclonal antibody Tan20 (ref. 6) (6% SDS-PAGE). TMIC identifies the furin-generated mature Notch-1 fragment. **c**, MEF cell extracts were reacted with mN1A, a monoclonal antibody against Notch-1 (4–20% SDS-PAGE, 50 μ g per lane).

release NICD^{7–9}. As endogenous NICD levels in cells and tissues are biochemically below the level of detection⁷, we took advantage of two well-characterized Notch constructs, mNotch Δ E and N^{ICV}, that have been used to demonstrate the relationship between processing of and signalling through Notch-1 (refs 7, 13). The mNotch Δ E construct codes for the transmembrane and intracellular domains of murine Notch-1 and undergoes a single proteolytic cleavage to release NICD. This cleavage resembles cleavage of endogenous Notch-1 in all respects^{7,13}. The N^{ICV} construct encodes the intracellular domain of Notch-1 without the membrane anchor, producing a protein identical to NICD. N^{ICV} has been used as a control for the fate of NICD in PS1^{-/-} cells⁷. Both constructs have part of their C-terminal domain replaced with six consecutive Myc-epitope tags⁷. We cloned these constructs into recombinant Semliki forest virus (SFV) vector, to enable their expression in primary cultures of neurons⁴. The expression and subcellular localization of mNotch Δ E were similar in wild-type and PS1^{-/-} cells, as was also the case for N^{ICV} (Fig. 3). However, NICD protein produced from N^{ICV} translocated to the nucleus in both PS1^{+/+} and PS1^{-/-} neurons (Fig. 3, lower panels), whereas nuclear staining in wild-type and PS1^{-/-} neurons expressing mNotch Δ E was almost undetectable, indicating that the relative amounts of NICD compared with unprocessed mNotch Δ E were below the detection limit of our immunofluorescence assay (Fig. 3, upper panels). These observations are in agreement with genetic evidence from *C. elegans* that *sel-12* mutations do not interfere with *lin-12* signalling when the LIN-12 intracellular domain is expressed alone¹¹.

To assay directly the role of PS1 in mNotch Δ E cleavage, we performed pulse-chase experiments and analysed the generation of NICD. Within 30–60 minutes after labelling with ³⁵S-methionine, NICD was first detected in PS1^{+/+} cultures. NICD continued to accumulate throughout the 120-minute chase period (Fig. 4a, b; ratio of NICD to total mNotch Δ E present at the start point of the chase). In contrast, NICD production was greatly reduced in PS1^{-/-} neurons (Fig. 4a, b). As the PS1^{-/-} embryos tend to yield fewer cells than their wild-type littermates⁴, we analysed twice the amount of PS1^{-/-} cell extracts. Only residual amounts of NICD were detected in PS1^{-/-} neurons (Fig. 4c), even when the mNotch Δ E signal was stronger than in the PS1^{+/+} cell extracts. This difference could not be explained by a higher turnover rate of NICD in PS1^{-/-} neurons, as NICD produced from control N^{ICV} constructs showed the same stability in both PS1^{-/-} and PS1^{+/+} cells (Fig. 4d).

To test the generality of this observation in another, non-neuronal, cell type, we transiently transfected PS1^{-/-} fibroblasts with mNotch Δ E plasmids. Although NICD was produced in PS1^{+/+}

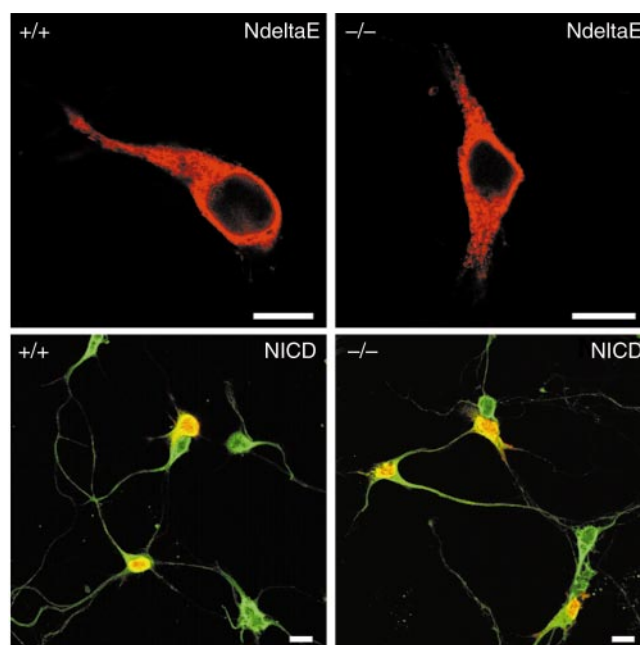


Figure 3 Nuclear transport of the intracellular domain (NICD) of Notch-1. Neurons were transfected with SFV driving expression of mNotch Δ E or N^{ICV} (which is identical to the NICD), fixed and immunostained. Red fluorescence indicates Myc-tagged mNotch Δ E (upper panels) or NICD fragment (lower panels). Nuclear import of NICD is not disturbed in the PS1^{-/-} neurons. The green fluorescence in the lower panels indicates immunoreactivity towards calnexin, revealing the ER. The presence of uninfected neurons in the lower panels shows the specificity of the anti-Myc staining. +/+, PS1^{+/+} neurons; -/-, PS1^{-/-} neurons.

cells, it was hardly detectable in PS1^{-/-} cell extracts (Fig. 4e, extract). Co-immunoprecipitation of NICD with its nuclear partner, Flag-tagged CSL^{RBp3} (ref. 7), confirmed the existence of very low amounts of NICD in the PS1^{-/-} cells (Fig. 4e). As residual amounts of both NICD and amyloid- β peptide can be detected in PS1^{-/-} cells, it is possible that the PS1 homologue PS2 (expressed in PS1^{-/-} cells; K.C. and B.D.S., unpublished observations), can partially compensate for the loss of PS1 function. We conclude that NICD production is decreased markedly in PS1^{-/-} cells.

The site of cleavage of Notch-1, releasing NICD, may occur at an analogous position to the site of cleavage of APP by γ -secretase¹⁴, N-terminal to the RKRRRQ stop-transfer signal⁷. This apparently

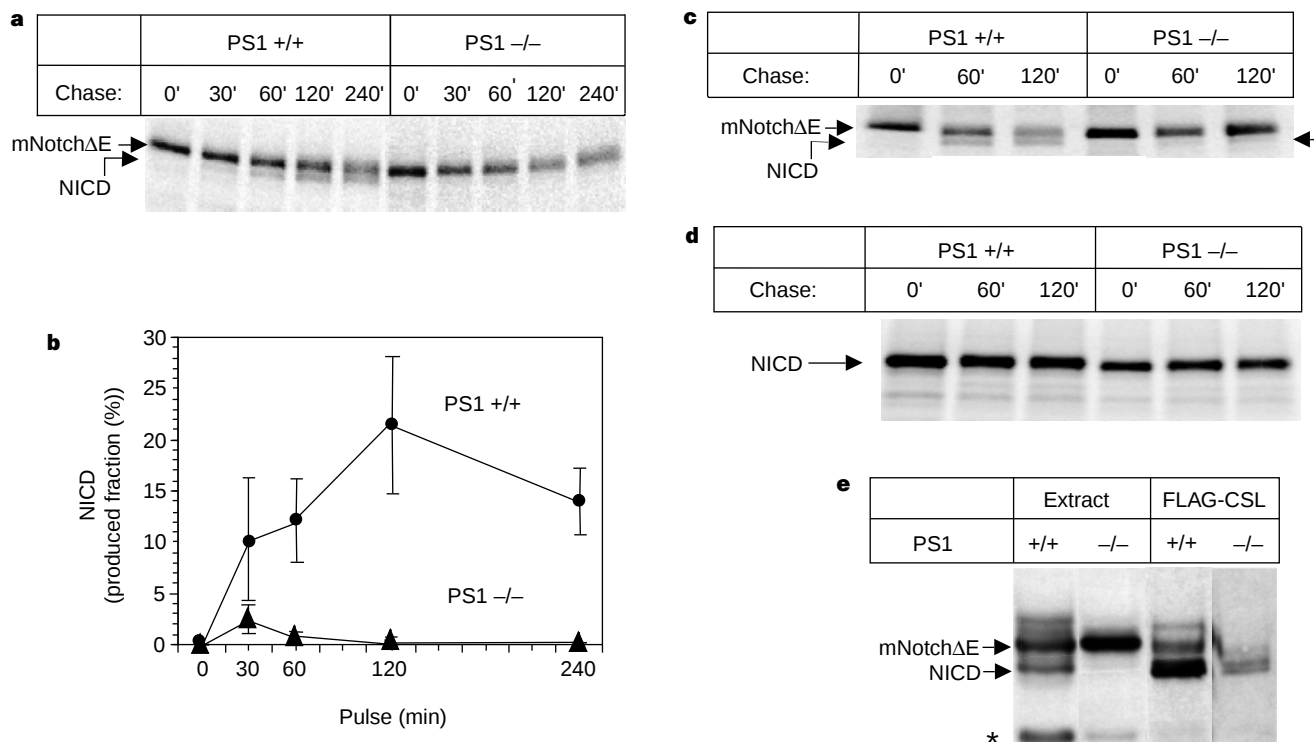


Figure 4 Generation of NICD in PS1^{+/+} and PS1^{-/-} neurons. **a**, Neurons transfected with SFV encoding mNotchΔE were labelled for 30 min and then chased in normal medium as indicated. Immunoprecipitated material was resolved in 4–20% SDS-PAGE. **b**, Phosphorimaging analysis of experiments performed as in **a**. The mNotchΔE signal at time zero was taken as 100% (lanes labelled 0' in **a**). NICD produced from mNotchΔE at each time point was normalized to this signal (produced fraction). Values are means ± s.e.m. (*n* = 3). **c**, Control experiment, performed as in **a** but using twice as much material from PS1^{-/-} neurons. NICD is hardly detectable at 120 min or 240 min of chase in PS1^{-/-} neurons (left arrow) even

when more mNotchΔE precursor is loaded. **d**, Turnover of NICD is identical in PS1^{+/+} and PS1^{-/-} neurons transfected with SFV encoding N^{icv}. The neurons were labelled for 30 min followed by a chase as indicated. **e**, mNotchΔE was expressed in PS1^{+/+} and PS1^{-/-} MEFs. We then analysed MEF extracts (left) and immunoprecipitates produced using anti-Flag-CSL antibodies (right) by western blotting. NICD is present in PS1^{+/+} cell extracts and undetectable in PS1^{-/-} cell extracts. Enriching for NICD using anti-CSL co-immunoprecipitation reveals residual amounts of NICD in PS1^{-/-} cells (5 min exposure, compared to 20 s in the other lanes). Asterisk indicates a post-lysis degradation peptide.

intramembranous cleavage is unusual and occurs in only one other known protein, the sterol-regulatory-element-binding protein (SREBP)¹⁵ (Fig. 1). However, mutants deficient in SREBP cleavage show no abnormality in generation of amyloid-β peptide, indicating that the enzyme that mediates SREBP cleavage may not be closely related to γ-secretase¹⁶. No effects on SREBP processing were observed in PS1^{-/-} cells (data not shown). In contrast, our results from PS1^{-/-} cells show that PS1 is important for cleavage of both Notch-1 and APP. To determine whether the enzyme responsible for NICD release has similar pharmacological properties to γ-secretase, we compared the effects of protease inhibitors on processing of Notch-1 and APP, selecting inhibitors that have been shown previously to block γ-secretase^{17–20}. These inhibitors also interfere with mNotchΔE processing (Fig. 5 and refs 7, 13). MDL 28170, which is a peptide aldehyde calpain inhibitor, was the first inhibitor shown to interfere with γ-secretase¹⁸, and MG132, another peptide aldehyde, also inhibits γ-secretase. However, MG132 (and possibly MDL 28170) are relatively broad-spectrum protease inhibitors that inhibit the proteasome. We ruled out the possibility that inhibition of the proteasome is involved in the observed effects of MDL 28170 and MG132 on Notch-1 processing by using lactacystin, a specific proteasome inhibitor, which has no effect on NICD generation (Fig. 5 and E.H.S. and R.K., unpublished observations). Likewise, lactacystin does not affect γ-secretase-mediated cleavage of APP²¹.

To further explore the apparent similarity between γ-secretase and the Notch-1 protease, we used the γ-secretase inhibitor MW167, a difluoro ketone peptide analogue designed on the basis of the primary sequence of the site of cleavage by γ-secretase in

APP¹⁷. This compound inhibited, in a concentration-dependent manner, γ-secretase-mediated cleavage of human APP expressed in neurons using recombinant SFV (Fig. 6), leading to the accumulation of APP C-terminal stubs and the inhibition of amyloid-β-peptide secretion into the medium. As both α- and β-secretase-processed fragments appeared in the cells (α- and β-stubs, Fig. 6) and as secretion of the APP ectodomain into the medium was unaffected (data not shown), neither α- nor β-secretase enzymatic activity is inhibited by MW167 (see also ref. 17). Processing of mNotchΔE was inhibited by MW167 at concentrations similar to those that inhibit APP processing (Fig. 6). The estimated half-maximal inhibitory concentration (IC₅₀) for MW167 (10 μM) in mNotchΔE processing is nearly identical to its IC₅₀ for APP¹⁷. MW167 is also a potent inhibitor of cathepsin D²²; however, using a series of related difluoro ketone analogues, we found that the effect of MW167 on APP processing is not a consequence of cathepsin-D inhibition²². The three inhibitors (MG132, MDL28170 and the chemically different MW167) all inhibit mNotchΔE processing and γ-secretase-mediated cleavage of APP to similar extents. Although neither γ-secretase nor the enzyme that cleaves mNotchΔE has been purified yet, our results are consistent with the hypothesis that these two proteolytic activities are closely related.

We have shown that efficient processing of Notch-1 to produce NICD requires PS1 and is inhibited by γ-secretase inhibitors. We showed this by using the mNotchΔE construct, a reliable model for intracellular processing of Notch-1 (ref. 7). Furthermore, the expression of endogenous Notch-1, its maturation by a furin-like enzyme, and the translocation of NICD to the nucleus are not affected by PS1 deficiency. Notch-1 and APP both exhibit a genetic

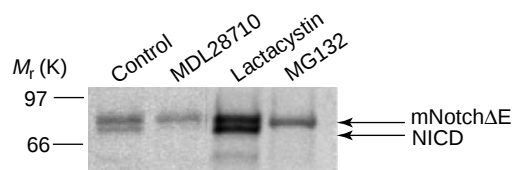


Figure 5 Effect of γ -secretase inhibitors on mNotch Δ E processing. Neurons were transfected with SFV encoding mNotch Δ E as in Fig. 4a, c. Label was chased for 4 h in the presence of 1% DMSO (control), MDL 28170 (100 μ M), lactacystin (100 μ M) or MG132 (20 μ M). The stronger signals in the lactacystin lane probably reflect stabilization of mNotch Δ E when the proteasome is inhibited.

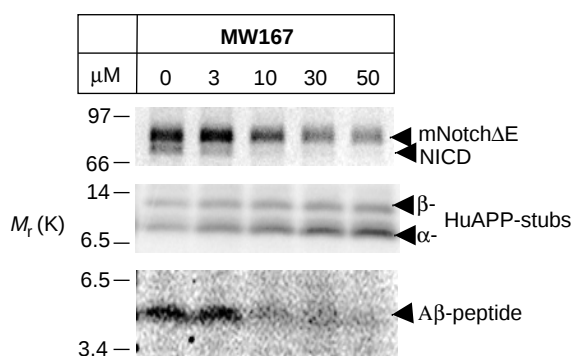


Figure 6 Effect of γ -secretase inhibitor MW167 on processing of mNotch Δ E (upper panel) and APP (lower panels). SFV-infected neurons were pulse-labelled for 30 min and label was chased for 4 h in the presence of DMSO vehicle or the indicated concentrations of MW167. mNotch Δ E and NICD were immunoprecipitated using anti-Myc antibodies. APP stubs (reflecting α -secretase- and β -secretase-mediated processing of human APP) and amyloid- β peptide (A β peptide; 4 h labelling) were immunoprecipitated as described^{4,12}.

interaction with presenilins^{1–3,11} and are cleaved by a γ -secretase-like activity that requires PS1 protein to perform an apparently intra-membranous cleavage. These results provide a molecular explanation for the widely reported genetic interaction between Notch-1 and PS1 (refs 1–3, 11) in different species.

There is also a direct physical interaction between Notch and PS1 (ref. 23), as there is between APP and PS1/2 (refs 24, 25). The function of PS1 in Notch/APP processing could be that of a 'facilitator', analogous to the role of the endoplasmic-reticulum (ER)-resident, multimembrane-spanning protein SCAP in SREBP¹⁵ processing. Such a facilitator could directly expose APP and Notch-1 to the γ -secretase. Alternatively, PS1 could indirectly regulate the correct trafficking of APP, Notch-1 and γ -secretase to an as-yet-undefined subcellular (micro) domain where cleavage occurs²⁶. However, our results are also consistent with a more direct role for PS1 as a regulatory or catalytic component of the protease(s) that cleave(s) Notch-1 and APP. In this case, PS1 would have a role similar to that of the ER-resident multimembrane-spanning site-2 protease²⁷ in SREBP processing (Fig. 1).

Finally, our results have significant implications for the potential use of γ -secretase inhibitors as drugs for treatment of Alzheimer's disease. The resulting inhibition of Notch-1 processing in the adult would be predicted to lead to immunodeficiency and anaemia because of the important function of Notch-1 in haematopoiesis throughout life^{28,29}. Thus, it may be necessary to develop inhibitors specific for γ -secretase-mediated cleavage of APP or to target other steps in the generation and deposition of amyloid- β peptide. □

Methods

The generation and characterization of PS1^{-/-} mice, the generation of neuronal cell cultures, the use of recombinant SFV, metabolic labelling, cell extraction and double immunoprecipitation were done as described^{4,12}. For pulse-chase experiments, cells from one embryo were seeded in a 12-well or 4-well cell-

culture plate (Nunc) pretreated with polylysine and serum. After infection with the appropriate recombinant SFV^{4,12}, neurons were pulse-labelled for 30 min with 200 μ Ci ³⁵S-methionine per ml, and chased for the indicated time in normal medium. Cells were processed as before¹². Every lane (Figs 4a, c, d, 5, 6) shows the material obtained from one well, and signals were compared between littermate embryos. MG132 (Calbiochem), MDL 28170 (provided by B. Cordell) and MW167, each dissolved in dimethylsulphoxide (DMSO), were added at the end of the pulse labelling to the indicated final concentrations. DMSO in the cell cultures was 1%.

Mouse brain was homogenized in 5 mM Tris-HCl, pH 7.4, 250 mM sucrose, 1 mM EGTA, 5 mM EDTA, 1 μ g ml⁻¹ pepstatin A and 100 U ml⁻¹ aprotinin, and centrifuged at 500g for 10 min. The resulting supernatant was centrifuged at 100,000g for 60 min. The pellet was resuspended in PBS and protein concentration was measured (Biorad protein assay).

We plated 5×10^5 mouse embryonic fibroblasts (MEFs) immortalized with SV40 large T antigen in 100-mM plates and transfected them by the Ca₃(PO₄)₂/BBS method¹³ with both Flag-CSL^{RBP3} and mNotch Δ E. After lysis in co-immunoprecipitation buffer⁷ containing 200 mM KCl, 10% of the sample was used for crude extract analysis. The remainder was co-immunoprecipitated⁷ using anti-Flag antibody. The washed pellet was resuspended in 50 μ l Laemmli lysis buffer and resolved by 6% SDS-PAGE, transferred to nitrocellulose membrane and visualized using 9E10 anti-Myc antibody^{7,13}. For immunocytochemistry, neurons cultured on glass coverslips were fixed in 4% paraformaldehyde and permeabilized in ice-cold methanol and acetone. Antibodies were diluted in blocking buffer and cover slips were mounted in Mowiol (Calbiochem). Cells were analysed using a MRC1024 confocal microscope (Biorad).

The complementary DNAs coding for mNotch Δ E and N^{ICV7,13}, antiserum APF675-695 (ref. 12) used to immunoprecipitate the APP C-terminal stubs, and antiserum APF597-612 (ref. 12) used to immunoprecipitate amyloid- β peptide have been described. We used mNotch Δ E in which Met 1727 was mutated to Val to prevent alternative translation initiation. NICD production from mNotch Δ E is unaffected by this mutation, as shown previously^{7,13}. B19/2 was raised against amino acids 30–44 of PS1. IC-antiserum against the cytoplasmic domain of murine Notch-1 (ref. 15) was provided by A. Israël and used to immunoprecipitate endogenous Notch-1 from brain extracts. Rat monoclonal antibody Tan20 (provided by S. Artavanis-Tsakonas) was used for immunodetection of precipitated Notch-1 (ref. 6). mN1A, a monoclonal antibody specific to the cytoplasmic domain of Notch-1 (provided by L. Milner), was used for detection of mouse Notch-1 from MEF SDS extracts. Calnexin antiserum and the anti-Myc monoclonal antibody 9E10 were provided by A. Helenius and J. Cremers.

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Presenilin is required for activity and nuclear access of Notch in *Drosophila*

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Presenilins are membrane proteins with multiple transmembrane domains that are thought to contribute to the development of Alzheimer's disease by affecting the processing of β -amyloid precursor protein¹. Presenilins also facilitate the activity of transmembrane receptors of the LIN-12/Notch family^{2–5}. After ligand-induced processing, the intracellular domain of LIN-12/Notch can enter the nucleus and participate in the transcriptional control of downstream target genes^{6–9}. Here we show that null mutations in the *Drosophila Presenilin* gene abolish Notch signal transduction and prevent its intracellular domain from entering the nucleus. Furthermore, we provide evidence that presenilin is required for the proteolytic release of the intracellular domain from the membrane following activation of Notch by ligand.

β -Amyloid precursor protein (β -APP) is a transmembrane protein that travels by way of the endoplasmic reticulum and Golgi to the cell surface and undergoes proteolytic processing (reviewed in ref. 1). A set of β -amyloid (A β) peptides are generated from β -APP by proteases known as the β - and γ -secretases. The β -secretase cleavage occurs in the extracellular domain and the heterogeneous γ -secretase cleavages in the transmembrane domain. Dominant mutations in either of two human *Presenilin* genes appear to cause Alzheimer's disease by increasing the amount of the A β 42(43) fragment that is produced. A null allele of mouse *Presenilin1* appears selectively to reduce γ -secretase activity¹⁰. These observations indicate that presenilin either stimulates the activity of γ -secretase, or is itself a component of γ -secretase¹⁰.

LIN-12/Notch proteins act as transmembrane cell-surface receptors for intercellular signals during development. It has been proposed that signal transduction involves cleavage and transport of the intracellular domain to the nucleus (reviewed in ref. 11), and results from experiments in *Drosophila*^{6,8,9} and mammalian cells⁷ strongly support this idea, indicating that cleavage occurs in or near the transmembrane domain. In mammalian cells, at least one

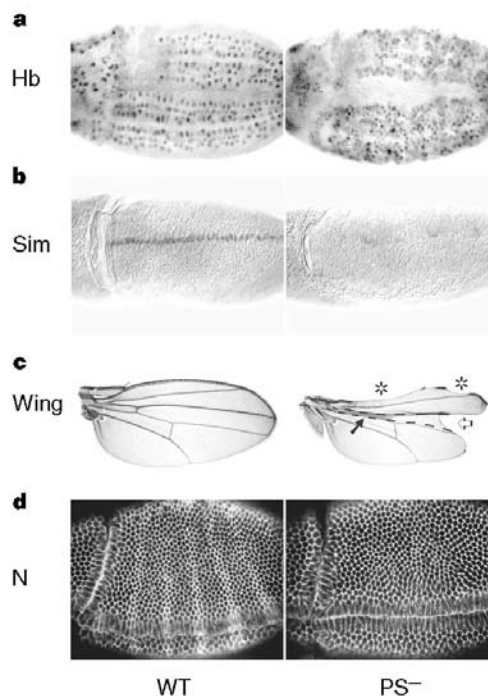


Figure 1 Presenilin activity is required for Notch activity. **a**, Hunchback (Hb) expression marks neuroblasts. Notch activity normally prevents more than one cell in a proneural cluster from segregating as a neuroblast. In *PS*[−] embryos, clusters of neuroblasts form instead of single neuroblasts, generating longitudinal bands, rather than lines, of neuroblasts. Hb expression is shown just after the completion of germ-band extension [the dorsal (top) and ventral (bottom) aspects of each embryo are shown in different focal planes; anterior is to the left]. **b**, Single-minded (Sim) expression marks ventral midline cells¹⁹. Notch activity normally promotes differentiation of midline cells. In *PS*[−] (and *N*[−]) embryos, relatively few of these cells arise, in contrast to wild-type embryos where they form a continuous two-cell wide column along the midline. Sim expression is shown dorsally, just after completion of germ-band extension. **c**, Right: a wing containing clones of *PS*[−] cells, marked with the *mwh* mutation (the *mwh* marker is not visible at this magnification). Notch activity is normally required to transduce signals between the dorsal and ventral compartments at the wing margin which are necessary for the survival and growth of wing blade cells²⁰. *PS*[−] clones that arise near the wing margin (asterisks) cause scalloping of the wing, as do *N*[−] clones in this region²⁰. Clones of mutant cells at a distance from the boundary can survive and populate internal portions of the wing blade (one such clone is indicated by an open arrow and outlined with dashed lines). However, such clones are associated with vein thickening (filled arrow), also attributable to a failure in signal transduction by Notch²¹. *PS*[−]/*PS*[−] embryos derived from *PS*[−]/*+* females can survive until the onset of pupation; however, the imaginal discs are abnormally small. **d**, Notch protein (N) accumulates to similar levels and is mainly associated with the plasma membrane in *PS*⁺ and *PS*[−] embryos. Both embryos are derived from *PS*[−] female germ cells and are shown ventro-laterally at the same stage of gastrulation with the presumptive Sim-expressing midline cells forming single cell columns flanking the ventral midline. Right: *PS*[−]/*PS*[−] zygote; left: *PS*[−]/*+* zygote, marked by the striped expression of β -galactosidase from a paternally derived third chromosome carrying both a *PS*⁺ allele and a *ftz-lacZ* reporter transgene (seven stripes of β -galactosidase expression can be seen superimposed on the otherwise normal pattern of N-protein expression). Such paternally rescued embryos can develop into normal first-instar larvae and give rise to adults that appear phenotypically wild-type.

proteolytic event occurs in the extracellular domain during Notch transit to the cell surface^{12,13}, and it has been suggested that ligand-binding might trigger additional extracellular proteolytic processing¹³. Thus, LIN-12/Notch proteins undergo proteolytic processing events that resemble the β - and γ -secretase cleavages of β -APP. These parallels, as well as genetic studies of presenilin in *Caenorhabditis elegans*, indicate that the presenilins may promote proteolytic cleavage during receptor maturation or activation^{6,14}.

To investigate the involvement of presenilin in proteolysis of LIN-12/Notch proteins, we wanted to use an *in vivo* assay for ligand-dependent cleavage and nuclear access of the intracellular domain that we developed in *Drosophila*⁶. We therefore identified null alleles of the single *Drosophila Presenilin* gene *PS* and examined their effects on the nuclear access and activity of Notch.

The *PS* gene has been identified by sequence analysis and mapped to a centromere proximal portion of the left arm of chromosome 3 (refs 15–17). To identify *PS* mutants, we examined a collection of recessive-lethal mutations that map to this location and cause a neurogenic phenotype in genetic mosaics (J. Jiang, C.-M. Chen and G.S., unpublished observations) for DNA sequence alterations in *PS*. Two independent mutations, *PS*^{C1} and *PS*^{C2}, contained lesions in *PS*. Both alleles are predicted to cause premature termination and appear to be null alleles: the predicted product of *PS*^{C1} lacks the last two transmembrane domains and other highly conserved sequences, and *PS*^{C2} contains a stop codon which truncates the coding sequence before the first transmembrane domain. *PS*^{C1} and *PS*^{C2} have indistinguishable phenotypes, and both alleles are fully complemented by the presence of a transgene expressing the *PS*⁺ coding sequence (see Methods), confirming that the phenotype is due to the alterations in the *PS* gene.

To generate embryos with no *PS* activity, we removed both maternal and zygotic *PS* activity by generating *PS*[−] embryos derived from *PS*[−] female germ cells. These embryos, referred to here as *PS*[−] embryos, appear identical to embryos without maternal and zygotic *Notch* activity (*N*[−] embryos). For example, in both *PS*[−] and *N*[−] embryos¹⁸, clusters of neuroblasts segregate at the positions nor-

mally occupied by single neuroblasts, as visualized by hunchback (Hb) staining (Fig. 1a). Both *PS*[−] and *N*[−] embryos also show extensive neural hyperplasia during subsequent development and die as pharate first-instar larvae lacking both dorsal and ventral cuticle (data not shown). In addition, the number of midline cells, as defined by the expression of *Single-minded* (*Sim*), is greatly reduced (ref. 19; Fig. 1b). Notch protein is found predominantly at the plasma membrane and at similar levels in both wild-type and *PS*[−] embryos (Fig. 1d, and data not shown). Hence, the profound developmental defects in *PS*[−] embryos appear to result from the absence of Notch signal-transducing activity, rather than from a marked decrease in Notch protein at the plasma membrane.

We have also generated clones of *PS*[−] cells in the imaginal discs, which give rise to the adult appendages, and find that these cells exhibit phenotypes similar to those of *N*[−] cells. For example, *PS*[−] clones in the wing cause scalloping and vein thickening (Fig. 1c), reflecting failures in well defined signalling events that depend on *Notch*, such as the specification of Wingless-secreting margin cells along the dorso-ventral compartment boundary²⁰ and the lateral inhibition of vein differentiation²¹.

The *PS*[−] phenotypes indicate that presenilin is essential for Notch function (see also ref. 3). *PS* null mutations do not significantly affect other aspects of *Drosophila* development, consistent with indirect evidence from *C. elegans* that the presenilins are not required for transport or processing of membrane proteins in general¹⁴.

We investigated the effect of *PS* null mutations on nuclear access by the Notch intracellular domain by using three Notch proteins in which the chimaeric transcription factor Gal4-VP16 (GV) is inserted in-frame into Notch (N) just after the transmembrane domain (at site 3; ref. 6). Nuclear access is assayed by *UAS-lacZ* expression⁶. *N*⁺-GV3 functions like the wild-type Notch protein and the intracellular domain gains nuclear access and has signal transducing activity only in the presence of the ligand, Delta (ref. 6; Fig. 2). *N*^{ECN}-GV3 contains a deletion that removes most of the extracellular domain and causes constitutive signal transducing activity and nuclear access in the absence of Delta (ref. 6; Fig. 2).

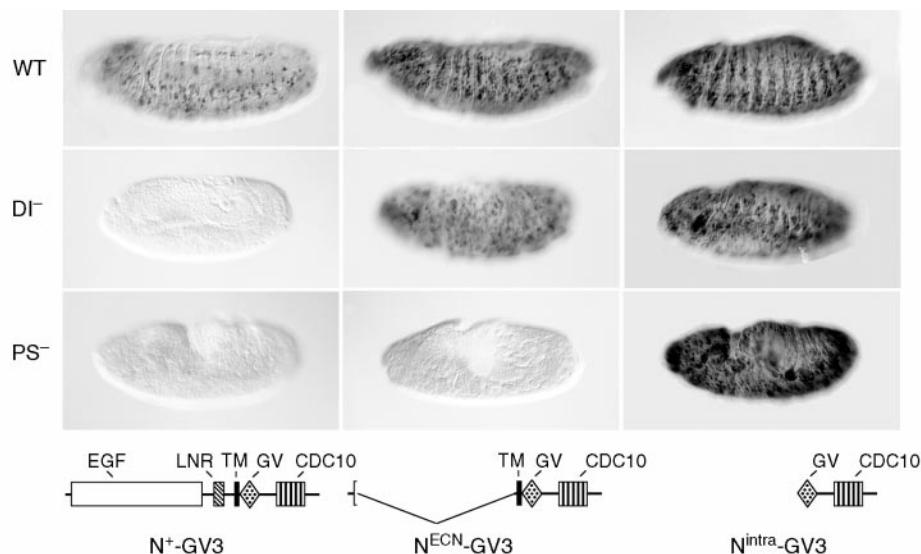


Figure 2 Presenilin is required for nuclear access of Notch. β -Gal expression indicates nuclear access of the Notch intracellular domain⁶. The N-GV3 constructs assayed are shown schematically at the bottom of each column. Wild-type Notch protein contains a region of 36 tandem EGF repeats (EGF), a region of three tandem LIN-12/Notch repeats (LNR), a single transmembrane domain (TM) and a region of six CDC10/SWI6/ankyrin repeats (CDC10). In all cases, the Gal4-VP16 domain (GV) is inserted at the beginning of the intracellular domain (site 3 in ref. 6). All embryos are shown in lateral aspect around the end of germ-band shortening (left: anterior). The same results were obtained using either the *PS*^{C1} or *PS*^{C2} allele.

N⁺-GV3 (left column): β -Gal expression is observed in wild-type (WT) but not in embryos lacking Delta (*D*[−]), indicating that nuclear access of the intracellular domain is ligand-dependent. β -Gal expression is not seen in *PS*[−] embryos (bottom), indicating that nuclear access requires presenilin. *N*^{ECN}-GV3 (middle column): Nuclear access is ligand-independent, as β -Gal is widely expressed in WT and *D*[−] embryos. However, no β -Gal expression is observed in *PS*[−] embryos, indicating that nuclear access requires presenilin. *N*^{intra}-GV3 (right column): There is widespread β -Gal expression in WT, *D*[−] and *PS*[−] embryos, indicating that nuclear access does not require presenilin.

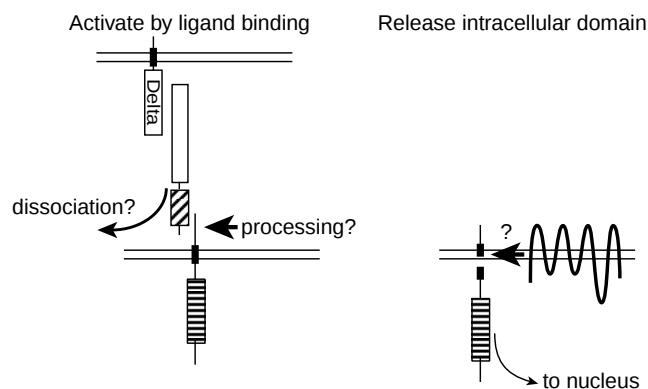


Figure 3 Model for LIN-12/Notch signal transduction. LIN-12/Notch proteins are represented as in Fig. 2, and presenilin is represented as an eight-transmembrane domain protein²³. Transmembrane protein ligands such as Delta, present on neighbouring cells, bind to LIN-12/Notch and activate signal transduction. We show the consequences of ligand-binding as two distinct phases, each of which may involve many events. The first phase is activation by ligand-binding, which may have one or more consequences. Ligand-binding may displace the amino-terminal portion of the heterodimeric receptor or otherwise lead to a conformational change. After ligand has bound, the receptor may dimerize^{27,28} or undergo processing of the extracellular domain¹⁹. The second phase is release of the intracellular domain, which involves proteolytic cleavage in or near the transmembrane domain. In principle, presenilin might be required for any event triggered by binding of ligand. However, presenilin is required both for ligand-independent access of N^{ECN} and ligand-dependent access of Nⁱ. We therefore favour the view that presenilin is required for the cleavage in the second phase. Presenilin might directly mediate cleavage within or near the transmembrane domain, perhaps by functioning as, or activating, a protease. Alternatively, presenilin may act indirectly, for example in the endoplasmic reticulum or Golgi, to facilitate the transport of other components needed for this cleavage.

N^{intra}-GV3 lacks the extracellular and transmembrane domains and also displays ligand-independent nuclear access (Fig. 2). The key difference between the two constitutively active forms is that N^{ECN}-GV3 retains the transmembrane and extracellular juxtamembrane domains whereas N^{intra}-GV3 is a cytosolic protein.

In *PS*⁻ embryos, neither N⁺-GV3 nor N^{ECN}-GV3 has access to the nucleus, as indicated by the complete absence of β -galactosidase (β -Gal) expression (Fig. 2). In contrast, the nuclear access of N^{intra}-GV3 is unaffected by the absence of presenilin activity (Fig. 2). The N⁺-GV3 observation indicates that presenilin activity is normally required for nuclear access of the Notch intracellular domain. Furthermore, the observation that presenilin is needed for nuclear access of N^{ECN}-GV3, a constitutively active transmembrane form, but not for N^{intra}-GV3, a constitutively active cytosolic form, suggests that presenilin participates in the release of the intracellular domain from the plasma membrane. Only about 35 amino acids of the Notch extracellular juxtamembrane region remain in the N^{ECN}-GV3 protein. Thus, if there are specific signals required for presenilin-dependent cleavage, they are likely to be somewhere in this region or within the transmembrane domain.

Although we have demonstrated that presenilin is necessary for the ligand-dependent nuclear access of the intracellular domain, we do not know whether presenilin directly mediates proteolytic release of the intracellular domain or if it acts more indirectly, for example by activating a protease or mediating its transit to the plasma membrane. Presenilin is found in a variety of cellular membranes, and β -APP may be processed at multiple sites, including the plasma membrane¹. Furthermore, in *Drosophila*, endogenous presenilin has been detected in the plasma membrane²², which is a likely site of ligand-dependent proteolysis.

Our findings can be incorporated into a model of events involved in LIN-12/Notch signal transduction, in which ligand-binding

activates LIN-12/Notch, thereby creating a substrate for presenilin-dependent release of the intracellular domain from the membrane (Fig. 3). Although there are other possibilities (Fig. 3 legend), release could require the direct participation of presenilin in the proteolytic cleavage of LIN-12/Notch proteins in or near the transmembrane domain. Presenilin may play an analogous role in the processing of β -APP¹⁰. □

Methods

Identification of *PS* mutations. Genomic DNAs were obtained from wild-type larvae and from *PS*⁻/*PS*⁻ larvae, and the *PS* genes were recovered by PCR amplification and sequenced. The *PS*^{C1} allele is associated with the insertion of 8 base pairs (bp) (GATATATA) in place of 2 bp (CG) immediately downstream of the codon Glu 438 (GAA) (numbered as in ref. 16). This insertion is predicted to destroy a splice donor and to truncate the protein before the last two transmembrane domains (according to the eight-transmembrane-domain topology model²³). The *PS*^{C2} allele is associated with a CAA → TAA mutation in the codon Gln52.

The *Tubulin α 1-PS*⁺ transgene was generated by placing the coding sequence of *PS* downstream of the *Tuba1* promoter¹⁸. Several independent transformants rescued both *PS* mutant alleles.

Generation of clones for phenotypic analysis. *PS*⁻ germline clones were obtained by the FLP/FRT/ovoD method²⁴ and females carrying these clones were fertilized by males carrying the same *PS*⁻ mutation in trans to a *TM3, ftz-lacZ* balancer chromosome, which allows paternally rescued zygotes to be distinguished from mutant zygotes. For the analysis of *PS*⁻ mosaics in the adult, clones of *PS*⁻ cells marked with *yellow* (*y*) and *multiple wing hairs* (*mwh*) were generated using the FLP/FRT method²⁵ by heat-shocking first-instar larvae of the genotype *y* *hs-FLP*; *mwh* *PS*^{C1} *FRT2A/DpSc*⁴, *y*⁺ *FRT2A*.

Transgenes and assay for nuclear access. The *UAS-lacZ* expression assay, the *hs-N*⁺-GV3 and *hs-N*^{ECN}-GV3 transgenes, and the results of expressing these transgenes in wild-type and *DL*⁻ embryos have been described⁶. The *hs-N*^{intra}-GV3 transgene is similar to the other two transgenes, except for the deletion of the upstream Notch coding sequence and the introduction of a start codon in front of the Gal4-VP16 coding sequence. To assay Notch nuclear access in *PS*⁻ embryos, females carrying *PS*⁻ germline clones were crossed to *UAS-lacZ/UAS-lacZ; PS*⁻ *hs-N*-GV3/*TM3, ftz-lacZ* males.

Protein localization Hunchback, Sim and β -galactosidase proteins were detected immunohistochemically^{6,18,19}. Notch was detected by immunofluorescence using a rabbit polyclonal antiserum directed against the intracellular domain⁹; predominant localization of Notch at the plasma membrane was confirmed by counterstaining with concanavalinA-Alexa594 (Molecular Probes) (data not shown).

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Neurogenic phenotypes and altered Notch processing in *Drosophila* Presenilin mutants

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Presenilin proteins have been implicated both in developmental signalling by the cell-surface protein Notch and in the pathogenesis of Alzheimer's disease. Loss of presenilin function leads to *Notch/lin-12*-like mutant phenotypes in *Caenorhabditis elegans*^{1,2} and to reduced *Notch1* expression in the mouse paraxial mesoderm³. In humans, presenilins that are associated with Alzheimer's disease stimulate overproduction of the neurotoxic 42-amino-acid β -amyloid derivative (A β 42) of the amyloid-precursor protein APP⁴. Here we describe loss-of-function mutations in the *Drosophila* Presenilin gene that cause lethal *Notch*-like phenotypes such as maternal neurogenic effects during embryogenesis, loss of lateral inhibition within proneural cell clusters, and absence of wing margin formation. We show that presenilin is required for the normal proteolytic production of carboxy-terminal Notch fragments that are needed for receptor maturation and signalling, and that genetically it acts upstream of both the membrane-bound form and the activated nuclear form of Notch. Our findings provide evidence for the existence of distinct processing sites or modifications in the extracellular domain of Notch. They also link the role of presenilin in Notch signalling to its effect on amyloid production in Alzheimer's disease.

To test directly the involvement of presenilin in Notch-receptor processing and signalling, we isolated lesions in the *Drosophila* Presenilin (*Psn*) gene by performing a lethal mutagenesis of the third chromosome region 77A-C uncovered by the deficiency *Df(3L)rdgC-co2*, an interval that harbours the *Psn* gene (see Methods). We recovered five lethal alleles of a single complementation group that is phenotypically rescued by genomic DNA fragments containing a functional *Psn* gene and which fails to be rescued by a DNA fragment from which the *Psn* coding region has been deleted (Fig. 1). All five *Psn* mutants have indistinguishable lethal phases and mutant phenotypes at the end of the larval period

when assayed in any heteroallelic combination or *in trans* to *Df(3L)rdgC-co2*, and so are likely to be strong or complete loss-of-function alleles. Mutant larvae secrete a pupal case and complete the last stages of larval development but do not form any adult structures; instead, they collapse into a homogeneous oily mass within the pupal case. This zygotic lethal null phenotype closely resembles that observed for *Suppressor of Hairless* (*Su(H)*), which encodes an effector protein of the Notch signalling pathway^{5,6}.

Formation of the dorsal/ventral (D/V) boundary along the presumptive wing margin requires Notch activity, and may be visualized by the expression of specific reporter genes in the margin zone of the late larval wing disc^{7–10}. Expression of *cut-lacZ* along the wing margin is abolished in *Psn* mutant wing discs (Fig. 2a, d). Similarly, margin zone expression of a *wingless-lacZ* construct and a *vestigial* D/V enhancer-*lacZ* transgene is absent in the *Psn* mutants, whereas *lacZ* expression outside the wing pouch persists in the mutants (Fig. 2b, c, e, f). Expression of a *vestigial* quadrant enhancer-*lacZ* transgene, which is specific to the non-margin zones of the wing pouch, is also completely absent in the *Psn*-mutant wing discs (Fig. 2g, j). Expression of these *wingless* and *vestigial* gene reporters along the wing margin and in the pouch region is directly dependent upon Notch signalling activity, and the *vestigial* D/V enhancer contains a critical binding site for the *Su(H)* protein¹¹. The absence of margin structures in the *Psn*-mutant wing discs, together with the overall reduction in size of the mutant wing pouch region, is reminiscent of conditional *Notch* mutant and *Su(H)* null mutant phenotypes^{5,7–10}.

A second feature of wing development that is mediated by *Notch* is the emergence of individual sensory organ precursor (SOP) cells at defined locations of the wing disc, a process that depends upon lateral inhibition among proneural cell clusters at each location to ensure that a single cell of the cluster adopts the proper SOP fate^{12,13}. Histochemical characterization of *Psn*-mutant wing discs using the early SOP markers *achaete-lacZ* and *scabrous-lacZ* reveals clusters of supernumerary SOP cells arising at certain normal SOP locations, as seen in typical neurogenic mutants (Fig. 2h, i, k, l). Lateral inhibition within these proneural cell clusters is severely impaired in *Psn*-mutant discs, resulting in enlarged SOP territories and increased proneural *achaete-lacZ* and *scabrous-lacZ* activity. To determine whether a similar requirement for presenilin in lateral signalling within the embryonic nervous system is masked by maternal deposition of the protein, we generated females with *Psn*-mutant germline clones (see Methods). Embryos derived from these females that lack both maternal and zygotic *Psn* activity

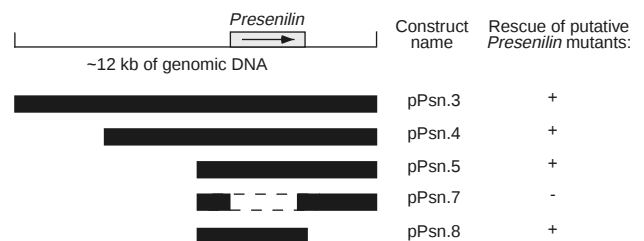


Figure 1 Identification of *Psn* mutations by genomic DNA rescue experiments. Transgenic flies were generated bearing different segments of wild-type genomic DNA from the *Psn* gene region and used to test different lethal complementation groups in the cytological interval 77A-C for phenotypic rescue to full viability. Construct pPsn.3 contains ~12 kb of genomic DNA, including the ~2.5 kb *Psn* transcribed region and ~7 kb and ~3 kb of 5' and 3' flanking sequences, respectively. Constructs pPsn.4 and pPsn.5 are similar to pPsn.3, except that they contain ~4 kb and ~1.2 kb of 5' flanking sequences, respectively. Construct pPsn.7 is identical to pPsn.5 except that it bears an internal deletion of most of the *Psn* gene extending from -23 bp (within the putative TATA box) to +2,331 bp (51 bp upstream of the stop codon). Construct pPsn.8, which extends from ~1.2 kb upstream of the *Psn* coding region to ~100 bp downstream of the *Psn* polyadenylation site, rescues only one of sixteen lethal complementation groups uncovered by *Df(3L)rdgC-co2*.

display a *Notch*-like lethal hyperplasia of the embryonic nervous system, and the maternal phenotype is only weakly rescued by wild-type paternal zygotic *Psn* function (Fig. 3).

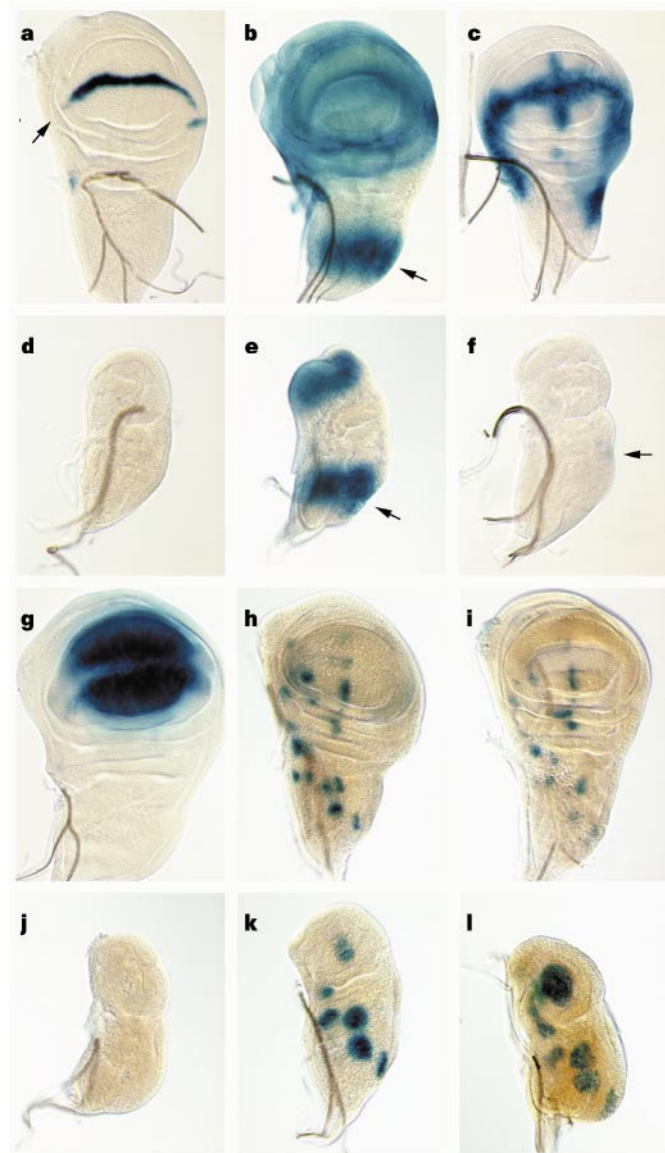


Figure 2 *Notch*-like phenotypes in *Psn* mutant imaginal wing discs. **a, d**, Expression of *cut-lacZ* in wild-type (**a**) and *Psn*^{B3}/*Df*(3L)*rdgC-co2* (**d**) wing discs, showing loss of *cut-lacZ* expression along the presumptive wing margin (arrow in **a**) in the mutant disc. **b, e**, Expression of *wingless-lacZ* in wild-type (**b**) and *Psn*^{B3}/*Df*(3L)*rdgC-co2* (**e**) wing discs, showing that *wingless-lacZ* expression is largely eliminated in the wing pouch region of the mutant disc (<20% of discs show complete loss of wing pouch expression) but appears normal in the notum region of the mutant disc (arrows in **b, e**). **c, f**, Expression of *vestigial* D/V boundary enhancer-*lacZ* in wild-type (**c**) and *Psn*^{B3}/*Df*(3L)*rdgC-co2* (**f**) wing discs, showing the loss of boundary-specific *vestigial* expression in the mutant disc, usually accompanied by residual expression in the notum area (arrow in **f**). **g, j**, Expression of *vestigial* quadrant enhancer-*lacZ* in wild-type (**g**) and *Psn*^{B3}/*Df*(3L)*rdgC-co2* (**j**) wing discs, revealing the complete loss of expression in the mutant disc. **h, k**, Expression of *achaete-lacZ* in wild-type (**h**) and *Psn*^{B3}/*Df*(3L)*rdgC-co2* (**k**) wing discs, showing elevated proneural regulatory activity and expanded SOP territories in the mutant disc. The strongly labelled proneural clusters in *Psn* mutant discs arise at reproducible positions and appear to correspond to the expanded proneural clusters detected in *Suppressor of Hairless* mutant wing discs⁵. **i, l**, Expression of *scabrous-lacZ* in wild-type (**i**) and *Psn*^{B3}/*Df*(3L)*rdgC-co2* (**l**) wing discs, confirming the elevated proneural activity and expanded SOP territories in *Psn* mutant discs. Anterior is at the bottom.

We investigated the role of presenilin in the expression and subcellular localization of Notch and its ligand Delta in view of evidence that presenilins influence the expression or facilitate the intracellular trafficking of these proteins in mice and worms^{1,2,4}. Third instar larval eye imaginal discs were chosen for this analysis because they exhibit a severe *Psn*-mutant phenotype and because both Notch and Delta show characteristic changes in protein levels and subcellular localizations in different eye disc regions^{14,15}. Parallel immunostaining of wild-type and *Psn*-mutant eye discs reveals that neither the accumulation nor the subcellular localizations of Notch and Delta are obviously altered in *Psn*-mutant eye discs, although subtle alterations may not have been detected. Notch is evident in an apparently normal apical membrane-associated and vesicular pattern throughout the disc, and shows the same increased accumulation in remnants of the morphogenetic furrow present in some *Psn*-mutant discs as in the wild-type furrow (Fig. 4a–f). These furrow remnants are associated with groups of cells expressing *sca-lacZ* or *atonal*, the *Achaete–Scute Complex* proneural gene for *Drosophila* photoreceptor development, which is similar to the raised proneural gene activities already noted for *Psn*-mutant wing primordia (Fig. 4f–j). Results were identical with antibodies recognizing either the extracellular or intracellular domains of Notch (data not shown; see Methods). Similarly, Delta protein expression levels and subcellular localization in *Psn*-mutant eye discs are comparable to wild-type (data not shown).

Drosophila and mammalian Notch is synthesized as an ~300K precursor protein that undergoes subsequent proteolytic processing

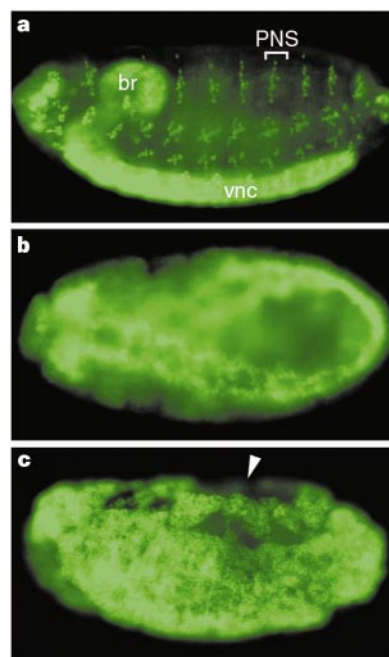


Figure 3 Neural hyperplasia in *Drosophila* embryos lacking maternal presenilin protein. **a**, Wild-type *Drosophila* stage-14 embryo immunostained with antibodies specific to the ELAV protein, which labels all neurons of the ventral nerve cord (vnc), brain (br) and segmentally arranged peripheral nervous system. **b**, ELAV-immunostained *Drosophila* embryo lacking maternally supplied presenilin, fertilized with a wild-type male sperm, exhibiting the disordered and expanded central nervous system characteristic of partially rescued maternal neurogenic mutants. **c**, ELAV-immunostained *Drosophila* embryo lacking maternal and zygotic presenilin, showing massive neural hypertrophy extending throughout the embryo except for a small dorsal patch (arrowhead), the typical phenotype for severe neurogenic mutant embryos. Zygotic *Psn* genotypes of individual maternally presenilin-deficient embryos were ascertained by immunostaining for the presence of either the wild-type *ftz-lacZ* marked balancer chromosome or the unmarked *Psn* mutant chromosome in fertilized eggs derived from irradiated *Psn*/*ovo*^{D1} females (see Methods). Anterior is on the left.

within the *trans*-Golgi network, such that mature Notch receptor present at the cell surface is a heterodimer consisting of the N-terminal extracellular EGF-homologous region joined to a smaller C-terminal Notch fragment consisting of a short extracellular stalk, the transmembrane domain, and the intracellular domain^{16–18}. This processing is thought to occur in two steps: first, a constitutive furin-mediated cleavage at a site ~70 amino acids N-terminal to the transmembrane domain^{17,19}; and second, a ligand-dependent cleavage at an extracellular site closer to the membrane^{17,18,20,21}, which may be mediated by the Kuzbanian/SUP-17 metalloprotease family^{17,22}. A subsequent intramembranous cleavage is thought to liberate a soluble intracellular Notch fragment that translocates to

the nucleus and activates transcription of Notch target genes, but which is produced at quantities too low to detect by conventional immunohistological methods^{18,21,23–25}.

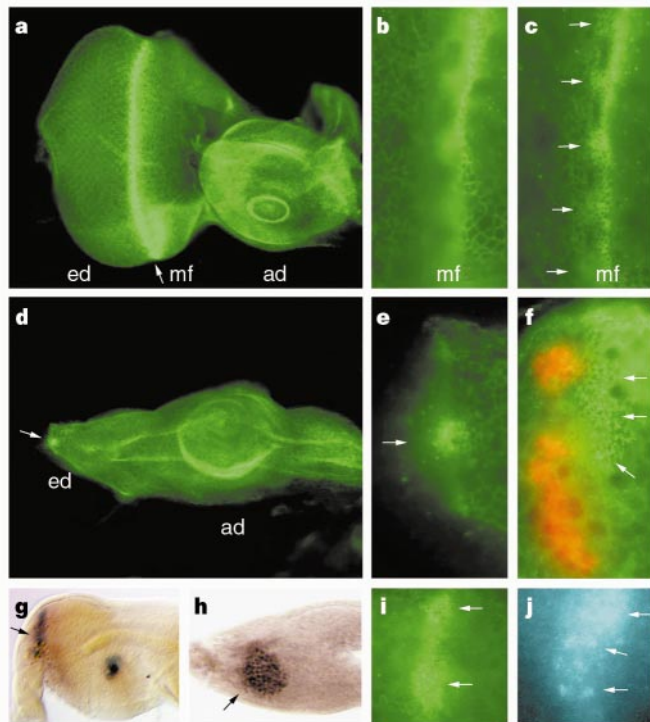


Figure 4 Immunohistochemical analysis of the Notch protein distribution in *Psn*-mutant eye imaginal discs. **a–e**, Notch expression and localization in wild-type and *Psn* mutant eye discs revealed by immunohistology using mouse anti-Notch mAb C458.2H. **a**, A wild-type eye disc exhibits relatively uniform Notch protein accumulation across the apical surface of the eye disc (ed) and antennal disc (ad) epithelia, with pronounced staining in the morphogenetic furrow (arrow, mf). **b, c**, High-magnification images of two focal planes of the morphogenetic furrow in **a**, revealing Notch protein localization to the apical cell membranes (**b**) and intracellular vesicles (**c**), as well as concentrated Notch accumulation in periodically spaced cell clusters within the furrow (arrows in **c**). **d**, An eye-antennal disc from a larva of genotype *Psn*^{B3}/*Df(3L)rdgC-co2* shows overall levels of Notch expression resembling wild-type, with similarly increased amounts of Notch in the furrow remnant in some *Psn* mutant eye discs (arrow). **e**, High-magnification view of the same furrow remnant as in **d**, showing apparently normal localization of Notch to apical membranes and vesicles, and high expression of Notch in apically constricted furrow-like cells (arrow) equivalent to that detected in wild-type furrow cells. **f–j**, Furrow-like cells in *Psn*-mutant eye discs are like wild-type furrow cells. **f**, Some neuronal differentiation, immunostained for the ELAV antigen (red), is detected in regions just posterior to the furrow remnants (arrows) of some *Psn* mutant eye discs. **g**, A small region of *sca-lacZ* expression (arrow), a furrow marker, is seen at the posterior tip of some *Psn*-mutant eye discs. **h**, Variably sized pockets of cells expressing *atonal*, another furrow marker, are detected in many *Psn* mutant discs. **i, j**, Double immunolabeling for Notch and Atonal demonstrates that furrow-like apical membrane Notch accumulation (**i**) is associated with underlying Atonal-positive cell nuclei (**j**) in these two focal planes of the same *Psn*-mutant eye disc sector. Anterior is on the right.

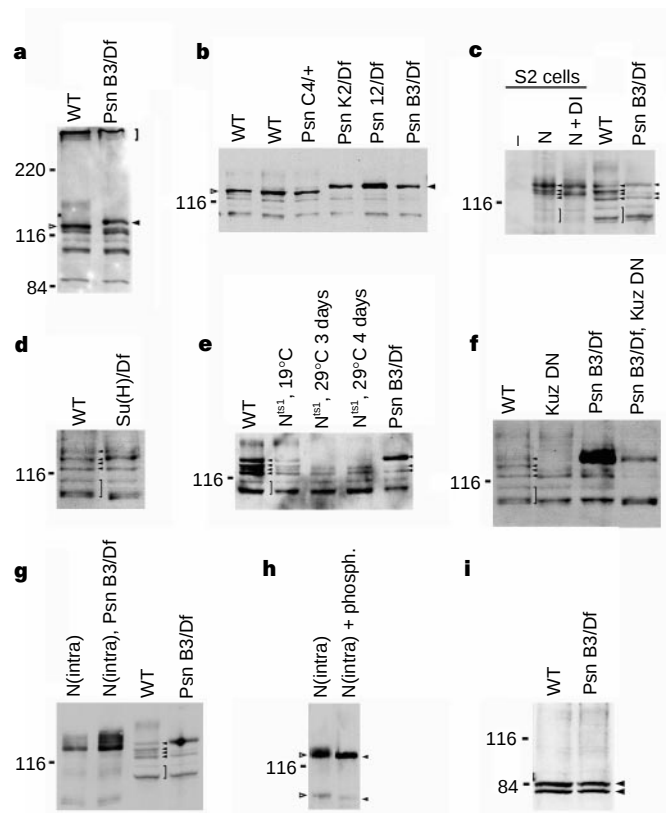


Figure 5 Western immunoblot analysis of Notch C-terminal fragments and Delta in *Psn* mutants. Third-instar larval brain-disc complexes of different genotypes and S2 cell lysates were analysed with mouse anti-Notch mAb C17.9C6. **a**, The most abundant C-terminal ~120K Notch fragment detected in *Psn*-mutant larvae (*Psn* B3/Df) migrates more slowly (black arrowhead) than the major ~120K Notch species in wild-type larvae (WT; white arrowhead), whereas ~300K Notch precursor amounts are equivalent (bracket). **b**, Smaller, ~120K forms (white arrowhead) predominate in wild-type and *Psn* C4/+ heterozygous larvae (*Psn* C4/+ het), whereas the largest C-terminal form (black arrowhead) is most abundant in three *Psn* mutants *in trans* to *Df(3L)rdgC-co2*. **c**, Untransfected S2 cell lysate (–), lysate of S2 cells transfected with full-length Notch cDNA²⁶ (N), lysate of aggregated Notch-expressing and Delta-expressing S2 cells²⁶ (N + DI), wild-type larvae, and *Psn*^{B3}/*Df(3L)rdgC-co2* larvae (*Psn* B3/Df). No obvious differences are seen in the overall ~120K Notch fragment profile (arrowheads, bracket) of Notch-expressing S2 cells (N) compared to Notch-expressing cells aggregated with Delta-expressing cells. **d**, Wild-type and *Psn*^{B3}/*Df(3L)rdgC-co2* (*Psn* B3/Df) larvae show similar Notch processing. **e**, Wild-type, *N*^{ts1} at the permissive temperature (19°C), *N*^{ts1} at the restrictive temperature (29°C) for 3 d and 4 d, and *Psn*^{B3}/*Df(3L)rdgC-co2* (*Psn* B3/Df) larvae, showing elimination of the two largest ~120K fragments in *N*^{ts1} at 29°C. **f**, Wild-type larvae, transgenic larvae overexpressing dominant-negative Kuzbanian²² (Kuz DN), *Psn*^{B3}/*Df(3L)rdgC-co2* (*Psn* B3/Df) and *Psn*^{B3}/*Df(3L)rdgC-co2* mutant larvae overexpressing dominant-negative Kuzbanian (*Psn* B3/Df, Kuz DN) have markedly reduced levels of the largest ~120K Notch fragment in the double *Psn* mutant, Kuz dominant-negative combination relative to the *Psn*^{B3} mutant alone. **g**, Larvae expressing the Notch intracellular domain²⁷ in a wild-type [*N*(intra)] or in a *Psn*^{B3} mutant background [*N*(intra), *Psn* B3/Df], compared to wild-type alone and *Psn*^{B3} mutant alone, revealing no effect of *Psn* on *N*(intra) mobility. **h**, *N*(intra) transgenic larvae either untreated [*N*(intra)] or treated with phosphatase [*N*(intra) + phosph], showing that some ~120K *N*(intra) forms (arrowheads, top) and a smaller Notch fragment (arrowheads, bottom) are likely to be phosphorylated. **i**, Wild-type and mutant *Psn*^{B3}/*Df(3L)rdgC-co2* (*Psn* B3/Df) larvae analysed with mAb C594.9B which recognizes Delta (arrowheads).

Western immunoblot analysis of larval brain-disc complexes from *Psn*-mutant larvae (>12 hours before death) demonstrates that Notch is aberrantly processed in the mutants. In wild-type larval tissues and S2 cells transfected with a *Notch* cDNA construct, the mouse antibody C17.9C6 against the intracellular domain of Notch²⁶ detects four major Notch C-terminal ~120K species and several smaller (<100K) Notch-derived fragments (Fig. 5a–c). In the *Psn* mutants, the largest ~120K form accounts for almost all of the ~120K Notch proteolytic products, whereas the second largest ~120K form is always almost or completely absent (Fig. 5a–c). This shift is unlikely to be a secondary effect of the *Psn* pupal lethal phenotype on Notch processing, because the pattern of ~120K Notch fragments is unaltered in identically staged *Su(H)*-null mutant larvae with an indistinguishable lethal phenotype (Fig. 5d). Moreover, reduced Notch activity in the conditional lethal allele *Notch^{ts1}* is characterized by an absence of the two principal ~120K processed fragments of Notch, instead of by overaccumulation of the larger of these fragments (Fig. 5e). Gel mobility of an intact intracellular Notch domain starting at an alanine located just C-terminal to the transmembrane domain²⁷ is unaffected in the *Psn*-mutant background (Fig. 5g, h), suggesting that phosphorylation or some other post-translational modification to the intracellular domain of Notch is unlikely to account for the altered pattern of endogenous ~120K Notch fragments in *Psn* mutants (Fig. 5a–c, e–g). In *Psn* mutants expressing a dominant-negative form of Kuzbanian lacking its protease domain, the production of all ~120K Notch C-terminal proteolytic fragments but not the smaller Notch fragments is severely reduced or eliminated, as has been reported previously for dominant-negative Kuzbanian alone²², although sometimes a small amount of the largest C-terminal fragment is detected (Fig. 5f). Overexpression of dominant-negative Kuzbanian may thus interfere with multiple extracellular Notch modifications and/or active furin synthesis, perhaps as a nonspecific steric effect^{17,18}, whereas mutant lesions in *Psn* may specifically block the more C-terminal modification without affecting the constitutive furin-mediated cleavage of Notch during receptor maturation in the *trans*-Golgi compartment. These results, together with the apparently normal subcellular localization of Notch in *Psn*-mutant tissues, agree with the finding that all Notch protein at the cell surface is in the furin-cleaved form^{16,17}. Ligand binding may cause further processing of the furin-cleaved Notch C-terminal fragment into a slightly smaller ~120K form that can represent a significant fraction of the total Notch C-terminal fragment pool^{17,18,20,21}. If this ~120K polypeptide is identical to the predominant ~120K Notch fragment that we detect in wild-type larvae, then its abundance in our samples suggests that it is unlikely to represent a ligand-induced proteolytic product. Alternatively, an unexpectedly large fraction of mature Notch might be ligand-bound in some tissues, so that the subsequent release of an active signalling domain may be inefficient. Western immunoblot analysis of Delta protein failed to reveal any effect of loss of presenilin activity on Delta protein levels or on its electrophoretic mobility (Fig. 5i).

We tested the ability of our *Psn* mutations to block the signalling activity of constitutively activated forms of Notch lacking the extracellular ligand-binding domain but containing different amounts of extracellular and transmembrane domain sequences in the vicinity of the putative cleavage sites. Ubiquitous expression of these different truncated Notch proteins that is driven by the heat-shock-inducible *hsp70* promoter suppresses neural precursor-cell specification in a ligand-independent manner^{27,28}. Consistent with our western immunoblot results, the constitutively activated, nuclear Notch intracellular domain N(intra), which lacks transmembrane sequences and the entire extracellular domain²⁷, is still able to signal in the absence of presenilin, as revealed by its ability to block neuronal specification of SOP cells in homozygous *Psn*-mutant wing discs (Fig. 6). Likewise, membrane-bound N-terminally truncated Notch proteins²⁸, which either lack all EGF

repeats but retain the Notch/Lin-12 repeats (Δ EGF) or which lack both the EGF and Notch/Lin-12 repeats (Δ ECN), also suppress SOP differentiation in *Psn*-mutant flies (Fig. 6). We cannot exclude the possibility that massive overexpression of truncated Notch allows inefficient cleavage and receptor activation by a physiologically irrelevant mechanism, although similar activated phenotypes have not been observed following comparable overexpression of full-length Notch²⁸. Our results make it improbable that presenilin is required specifically for the cleavage that occurs within or just C-terminal to the transmembrane domain and which has been proposed to liberate an active signalling domain that translocates to the nucleus^{21,23,24}. The production of such a nuclear Notch fragment, however, could depend indirectly upon the activity of presenilin in upstream events of Notch synthesis, heterodimer assembly, or ligand-induced activation. Both the biochemical and the genetic epistasis results presented here indicate that presenilin probably acts upstream of signal transduction by activated Notch before or during receptor activation by ligand binding, which is consistent with studies in *C. elegans*². For example, presenilin may be required for the proper assembly of furin-cleaved N- and C-terminal Notch fragments into functional heterodimeric receptors, for the subsequent extracellular cleavage of these heterodimers by Kuzbanian or another protease, or for some unknown post-translational modification to the Notch C-terminal domain.

Proteolysis of the human amyloid-precursor protein, which leads to the amyloid brain deposits characteristic of Alzheimer's disease, has similarities to Notch-receptor processing. APP is a single-pass transmembrane protein that is first cleaved at two alternative sites in

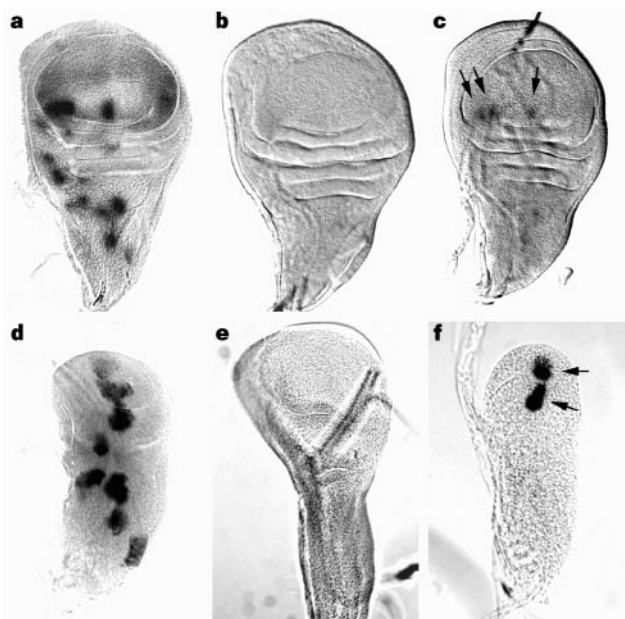


Figure 6 Effects of truncated, constitutively activated forms of Notch on SOP determination in *Psn* mutant tissues. **a, d**, Proneural cluster formation in wild-type (**a**) and *Psn^{B3}* mutant (**d**) control wing imaginal discs bearing no activated Notch transgenes but subjected to multiple head shocks (see Methods). **b, e**, Expression of a truncated Notch protein containing only intracellular domain sequences, termed N(intra)²⁷, in wild-type (**b**) and *Psn^{B3}*-mutant (**e**) wing discs completely suppresses SOP determination, as revealed by staining for proneural gene activity (see Methods). **c, f**, Expression of the membrane-tethered Notch truncation Δ ECN, which lacks all extracellular EGF and Lin-12/Notch repeats²⁸, in wild-type (**c**) and *Psn^{B3}*-mutant (**f**) wing discs, showing suppression of most SOP clusters in both genotypes. Occasional SOP formation in Δ ECN wing discs (arrows in **c, f**) is due to the weaker signalling activity of Δ ECN relative to N(intra). Results were similar with another membrane-bound Notch truncation, Δ EGF, lacking only the EGF-homologous extracellular domain of Notch²⁸ (not shown). Anterior is at the bottom.

the extracellular region just beyond the transmembrane domain, then at an unusual cleavage site within the transmembrane domain by as-yet undiscovered γ -secretase(s)²⁹. These events may be analogous to the extracellular Notch cleavages mediated by furin and perhaps by Kuzbanian, and a ligand-dependent subsequent cleavage within or just internal to the transmembrane domain. Mutant variants of presenilin that are associated with Alzheimer's disease alter the ratio of short and long β -amyloid produced by differential cleavage or carboxypeptidase activity at the γ position, generating more of the neurotoxic 42-amino-acid form relative to the less harmful 40-amino-acid form⁴. However, neurons from mice that are deficient in *Presenilin-1* have a relatively specific block in the γ -secretase cleavage of APP³⁰. The overaccumulation of the larger ~120K Notch C-terminal fragment in *Psn*-mutant flies may reflect a common role for presenilins in the regulation of APP and Notch processing, as the absence of presenilin results in both cases in the retention of extracellularly cleaved Notch or APP forms and the presumed inhibition of subsequent transmembrane cleavages. However, although presenilin is not required for either of the two extracellular cleavages of APP, it is needed for one of the two putative extracellular cleavages of Notch in *Drosophila*. Although we still do not know the exact role of presenilin in Notch processing, the *Notch*-like phenotypes and altered Notch protein species seen in *Drosophila Presenilin* mutants are suggestive of a shared mechanism of regulated proteolysis that may be a key feature of both Notch signalling and Alzheimer's disease. □

Methods

Drosophila genetics. Fly culture and crosses were performed according to standard procedures at 25 °C. To isolate mutations in the *Psn* gene, *w*¹¹¹⁸ males were fed 25 mM ethylmethane sulphonate and crossed to *y*¹ *w*¹¹¹⁸; *TM3* females. Individual *y*¹ *w*¹¹¹⁸; *TM3* male progeny of this cross were mated to *Df(3L)rdgC-co2/TM6C* females, and third chromosomes bearing lethal mutations uncovered by the deficiency were recovered in the progeny of this mating as balanced *TM6C* stocks. Screening of 5,160 mutagenized third chromosomes yielded a total of 79 lethal mutations uncovered by *Df(3L)rdgC-co2*. *Inter se* lethal complementation testing placed 70 of these mutations into 16 complementation groups, one of which consists of five independent *Presenilin* gene mutations termed *Psn*^{B3}, *Psn*¹², *Psn*^{C4}, *Psn*^{K2} and *Psn*^{S3}. To produce embryos deficient for maternal presenilin, eggs of genotype *Psn*^{S3}/*ovo*^D were collected for 24 h, aged for 48 h, and irradiated with 1,000 rad X-rays to induce germline clones. Presenilin-deficient embryos produced from homozygous *Psn*^{S3} germline clones were fertilized by *Psn*^{B3}/*TM3*; *fz-lacZ* males, allowed to develop for 24 h, then fixed and immunostained with antibodies against ELAV (for embryonic-lethal, abnormal vision) and β -galactosidase (see below) to distinguish between maternal *Psn*-null embryos lacking zygotic *Psn* and those with a paternally supplied wild-type *Psn* gene.

Genomic DNA rescue fragments. A ~12 kb *SacII/XhoI* genomic fragment containing the *Psn* transcription unit was subcloned from P1 phage clone DS02810 (Berkeley *Drosophila* Genome Project, personal communication) into pCaSpeR-4 to make pPsn.3. Partial *EcoRI* digestion of pPsn.3 gave pPsn.4 and pPsn.5, which contain a ~9-kb and ~6-kb *EcoRI/XhoI* genomic DNA insert, respectively. pPsn.7 is identical to pPsn.5, except that it bears an internal deletion of a *SpeI* fragment extending from -23 bp to +2,331 bp of the *Psn* gene. pPsn.8 contains a ~3.7-kb *EcoRI/BamHI* genomic DNA fragment extending ~1.2 kb upstream of the *Psn* transcriptional start site to ~100 bp downstream of the poly(A) addition site.

Histology and immunohistology. β -Galactosidase histological staining of wing discs has been described⁵. For immunohistology of *Drosophila* tissues, the following primary antibody dilutions were used: 1:200 rat anti-ELAV monoclonal antibody (mAb) 7E8A10 (gift from G. M. Rubin); 1:1,000 mouse anti-Notch mAb C458.2H against the extracellular domain of Notch^{14,26}; 1:1,000 mouse anti-Notch mAb C17.9C6 against the intracellular domain of Notch^{14,26}; 1:1,000 mouse anti-Delta mAb C594.9B against the extracellular domain of Delta^{15,26}; 1:4,000 rabbit anti-Atonal polyclonal antibody (gift from Y. N. Jan); and 1:30 mouse anti-Scabrous mAb sca1 (Developmental Studies Hybridoma Bank, University of Iowa). Fluorescence-conjugated secondary antibodies were used at the recommended dilutions (Jackson ImmunoResearch). Tissues were

mounted in 90% glycerol, 10% 1M Tris (pH 8.0), 0.5% *n*-propyl gallate, and images were collected using a Leica DRMB compound fluorescence microscope equipped with a cooled Hamamatsu C5810 CCD digital camera and imported into Adobe Photoshop 4.0. For experiments with N(intra), Δ EGF and Δ ECN, larvae were subjected to six alternating periods of 25 min 37 °C heat shock and 3 h 25 °C recovery periods, followed by fixation and staining at the wandering third instar stage.

Western immunoblot analysis. Western immunoblot analysis of larval brain-disc complexes was done as described²². Approximately six brain-disc complexes for endogenous Notch or one brain-disc complex for intracellular N(intra) were used for each SDS-PAGE gel lane. Larval extracts were treated with phosphatase as described²¹. Transfection and aggregation of S2 cells were performed as described²⁶. Western nitrocellulose transfer membranes were probed with mouse monoclonal antibodies C17.9C6 or C594.9B at a 1:1,000 dilution.

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The motor protein myosin-I produces its working stroke in two steps

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Many types of cellular motility, including muscle contraction, are driven by the cyclical interaction of the motor protein myosin with actin filaments, coupled to the breakdown of ATP. It is thought that myosin binds to actin and then produces force and movement as it 'tilts' or 'rocks' into one or more subsequent, stable conformations^{1,2}. Here we use an optical-tweezers transducer to measure the mechanical transitions made by a single myosin head while it is attached to actin. We find that two members of the myosin-I family, rat liver myosin-I of relative molecular mass 130,000 (M_r 130K) and chick intestinal brush-border myosin-I, produce movement in two distinct steps. The initial movement (of roughly 6 nanometres) is produced within 10 milliseconds of actomyosin binding, and the second step (of roughly 5.5 nanometres) occurs after a variable time delay. The duration of the period following the second step is also variable

and depends on the concentration of ATP. At the highest time resolution possible (about 1 millisecond), we cannot detect this second step when studying the single-headed subfragment-1 of fast skeletal muscle myosin II. The slower kinetics of myosin-I have allowed us to observe the separate mechanical states that contribute to its working stroke.

Structural studies have shown that some members of the myosin-I (brush-border myosin-I, BBM-I)³ and myosin-II families (chicken smooth-muscle myosin⁴) adopt at least two structurally distinct, bound conformations, one in the presence and the other in the absence of the bound nucleotide, ADP. These equilibrium experiments, together with dynamic mechanical experiments made using muscle fibres⁵, indicate that there may be one or more mechanical transitions as actomyosin breaks down a single molecule of ATP. We have used an optical-tweezers transducer⁶ to compare the mechanical properties of three different single-headed myosins: rat liver 130K myosin-I (the unspliced *myr-1a* gene product⁷, referred to here as myr-1), intestinal BBM-I, and a single-headed proteolytic sub-fragment of fast skeletal muscle myosin-II (S1). These myosins all have similar catalytic domains but possess regulatory domains that bind different numbers of light chains: S1 has two, BBM-I has three and myr-1 has six light chains⁸. In the experiments, a single actin filament was suspended between two plastic microspheres held in two optical tweezers. The actin filament was then positioned in the vicinity of a third, surface-bound microsphere that was sparsely coated with myosin. Actomyosin interactions were monitored by measuring the position of one of the microspheres holding the actin filament (Fig. 1).

To measure the working stroke produced by single actomyosin

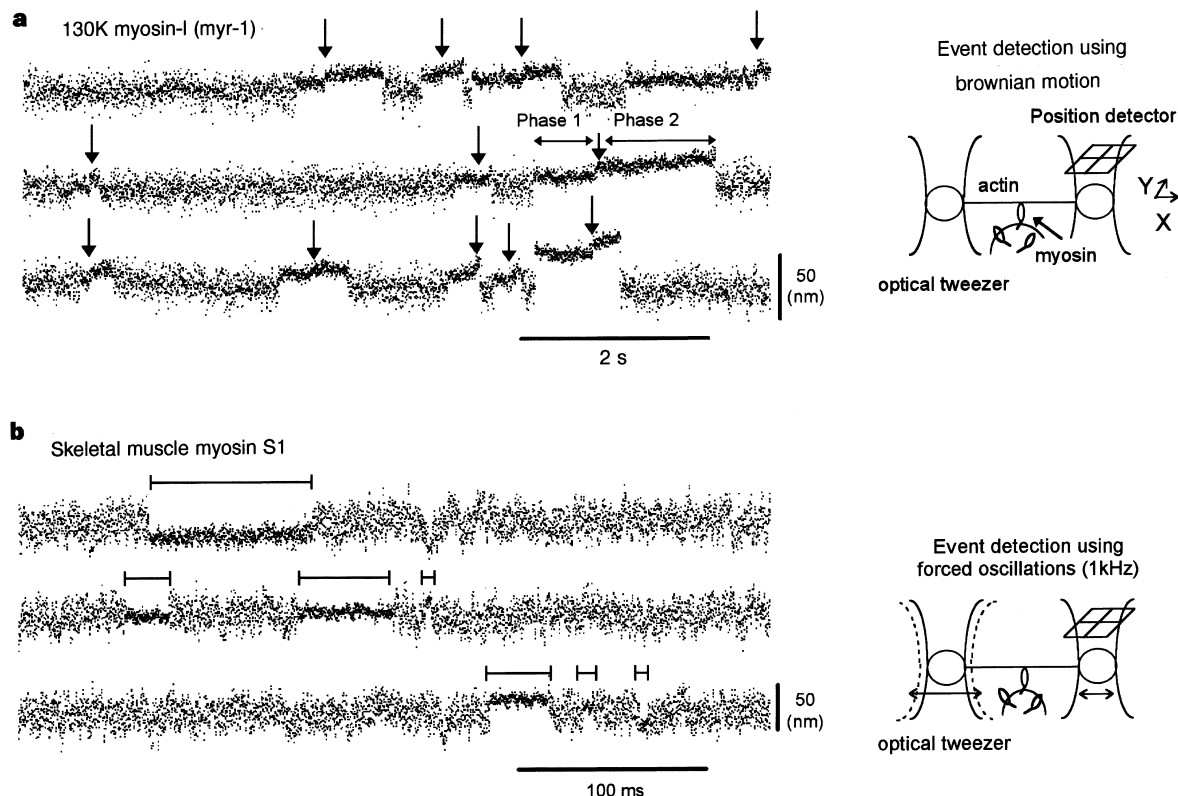


Figure 1 Single-molecule mechanical interactions measured for myr-1 and skeletal-muscle myosin S1. **a**, Three sequential displacement records for myr-1 interacting with rabbit skeletal actin (25 μ M ATP). Records show bead position measured parallel to the filament axis versus time. Each myosin-binding event causes a drop in thermal motion (see Methods). A feature of the records obtained with myr-1 (**a**) and BBM-1 (data not shown) is an additional stepwise change in bead position that occurs during the attached interval (vertical arrows). Steps

occur with a variable delay after the start of binding (phase 1) and the new position is maintained until the end of the interaction (phase 2). **b**, Steps are not apparent with skeletal muscle myosin S1 (10 μ M ATP). In this record, our ability to detect binding was improved by applying a 1-kHz oscillation to one of the optical tweezers and monitoring transmission of this signal to the other bead (see Methods). Attached intervals are indicated by solid black lines.

Table 1 Average lifetimes of myosin attachments

Myr-1 [ATP]	Phase 1			Phase 2		
	Mean (ms)	s.e.	(n)	Mean (ms)	s.e.	(n)
3 μ M	299.7	28.1	(114)	305.5	28.7	(113)
10 μ M	267.9	20.7	(166)	159.5	13.2	(143)
30 μ M	282.4	29.6	(91)	80.0	8.5	(89)
100 μ M	286.3	26.5	(115)	50.6	5.1	(96)
BBM-1						
5 μ M	110.7	7.9	(196)	100.9	7.2	(194)
50 μ M	120.7	8.6	(195)	44.8	3.6	(157)
Skeletal S1	One phase (only)					
	Mean (ms)	s.e.	(n)			
4 μ M	39.5	2.8	(204)			
10 μ M	21.5*	1.3	(269)			
50 μ M	8.3*	0.5	(332)			
100 μ M	7.0*	0.6	(149)			

We measured the lifetimes of phase 1 and phase 2 at different [ATP] and found that they were variable and fitted well to a single exponential time course. We have tabulated the average lifetimes, number of interactions (n), and their theoretical standard error²⁴ (where s.e. is defined as: $\sigma/\sqrt{n}$). Lifetime (τ) was given by $(t_{av} - t_{min})$, where t_{av} is the measured average for all events longer than t_{min} (the shortest event duration that could be determined reliably). We used $t_{min} = 40$ ms for events detected using analysis of brownian motion and $t_{min} = 4$ ms for events detected using the 1-kHz carrier oscillation (marked *). We could distinguish only a single phase for skeletal S1, which had an average duration that was strongly dependent upon [ATP].

interactions, the stiffness of the optical tweezers transducer must be significantly lower than the stiffness of the actomyosin complex and other series stiffnesses separating the trapped bead from the coverslip surface⁹. At such low tweezer stiffness (here 0.02–0.03 pN nm⁻¹), baseline thermal motion of the trapped beads is large (Fig. 1a and Methods) compared with the expected size of the working stroke². In a previous study⁶ we found that myosin can bind to actin over the full range of this thermal motion and that binding produces a characteristic reduction of brownian noise because of the increase in system stiffness. Because the mechanical system is overdamped (that is, viscous damping of the bead movement is large compared with the tweezer stiffness), detection of actomyosin binding requires about 10 ms. The properties of the transducer are important because we are interested in whether myosin produces movement immediately as it binds to actin or if there is a delay between binding and movement. Biochemical studies¹⁰ suggest that each mechanical interaction starts as myosin binds to actin with the products of ATP hydrolysis bound (actin + myosin.ADP.Pi). As phosphate is released from the complex there is a transition to a tightly bound, force-generating (actin.myosin.ADP) state^{11,12}. Subsequent loss of ADP leads to another tightly bound complex (actin.myosin), and additional movement may be generated during this process. The mechanical event is terminated when a new ATP molecule binds to myosin causing its detachment from actin (actin + myosin.ATP). If

the biochemical states are tightly coupled to force production then single-molecule studies should reveal movement occurring in one or more phases following a delay after actomyosin binding. In addition, the detachment rate should depend on the concentration of ATP.

Single-molecule mechanical records obtained using myosin-I showed two major differences from those obtained with skeletal muscle S1. First, the attachment durations were 20–50 times longer than those measured for S1 over a wide range of ATP concentrations (Table 1), consistent with the slower biochemical kinetics of BBM-1 (ref. 13) and myr-1 (ref. 7) compared to S1 (ref. 10) (particularly product release and ATP binding). Second, and most strikingly, both myr-1 (Fig. 1a) and BBM-1 (data not shown) produced movement in two phases. An initial motion of about 6 nm occurred within 15 ms of binding, and another stepwise change in position (of about 5.5 nm) occurred after a variable time delay (phase 1) from the onset of binding. The new position was maintained for another variable period (phase 2). The second step was not apparent in any of the records obtained with S1 (Fig. 1b). We measured the lifetimes of phase 1 and phase 2 (Fig. 1) for both myr-1 and BBM-1 and found them to be exponentially distributed (Table 1). Phase 1 was not greatly affected by the concentration of ATP ([ATP]), whereas phase 2 was significantly longer at lower [ATP]. The lifetimes of the single phase found for S1 and phase 2 for myr-1 were linearly dependent upon 1/[ATP], indicating that each event was probably terminated by binding of 1 ATP molecule.

Because the records are noisy, it is important to establish that the extra step is a consistent feature of the myosin-I data and to determine the amplitudes of each step. For this reason, we devised an averaging method that preserves the amplitude of the start and end position of each interaction, but which reduces the noise due to thermal motion (Fig. 2).

The averaged records (Fig. 3a) show that, after binding to actin, all three myosins produce a rapid displacement of about 5 nm. Data obtained using myr-1 and BBM-1 showed that the final position (just before detachment) was consistently shifted by a further 5–6 nm; S1 data did not show this property. The average size of the overall motion produced by the different myosins was 5.5 nm for S1, 11 nm for BBM-1 and 12 nm for myr-1 (after correcting by 10% for the effects of series compliance⁹). To check that the averaged data is homogeneous between records, we plotted running histograms (Fig. 3b) to show how the data are distributed. Because the thermal noise between records shows no temporal correlation, the running histograms have a gaussian distribution of positions at each time point, having a standard deviation that is similar to the noise of any

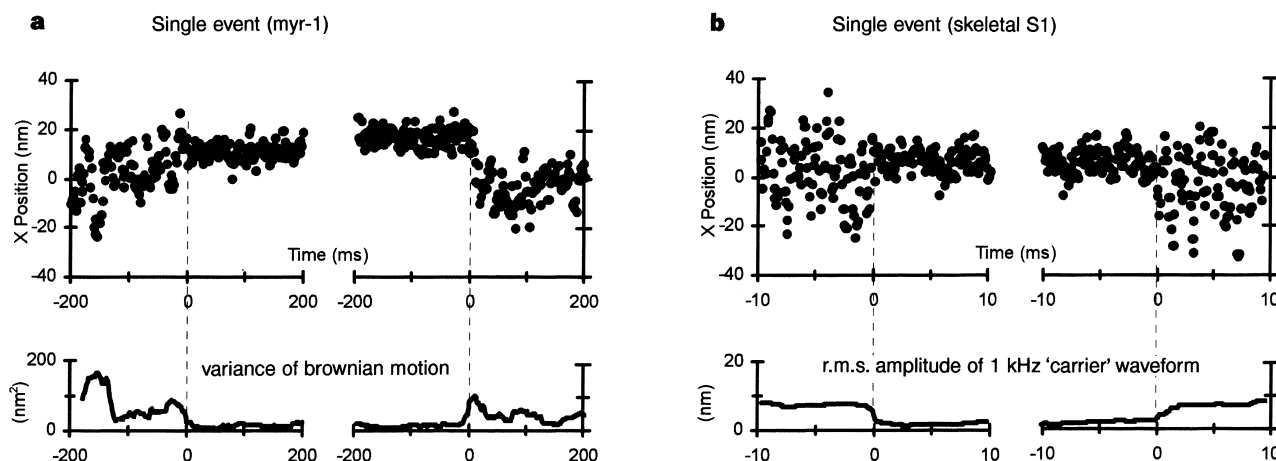


Figure 2 Averaging method to preserve amplitude of start and end of each interaction. **a**, Top, beginning and ending section of a single acto-myr-1 interaction. Timing of the start and end of each attachment (vertical dotted lines) was determined by thresholding the changes in variance of the brownian

motion (bottom). Because the overall duration of the events is random, the middle section of data, which is of variable duration, was removed. **b**, An attachment event produced by S1; the start and end of the attachment were determined from the change in amplitude of the 1-kHz carrier signal (dotted lines; see Methods).

single record when no myosin is bound (for example, the myr-1 data before and after attachment, s.d. = 8.5 nm); during the attached phases noise is greater because of additional system noise (for example, the myr-1 data during attached phases, s.d. = 9.3 nm). We analysed the step amplitudes for myr-1 by calculating the difference in mean position during phase 1 and phase 2 (measured for each event): mean = 5.5 nm, s.d. = 3.7 nm, $n = 63$. The same analysis on long attachments (containing no steps) showed: mean = 0.05 nm, s.d. = 2.1 nm, $n = 66$. The small difference in variance might be explained if the step size was of variable amplitude.

It is possible that a second step might also occur with S1 but that it might be either too small or too fast to be observed in any single record. We tested this idea using a new method to detect binding that has a much better time resolution than simply monitoring changes in brownian noise. We applied a high-frequency 'carrier' oscillation (at 1 kHz) to one of the optical tweezers and tracked the pickup of this signal by the bead held in the stationary tweezer (as described in Fig. 1b legend). We averaged many events (as before) and found that the rising phase required 5 ms to saturate, whereas the falling phase was complete within 1 ms (within the expected time resolution of the technique, Fig. 3a, lower). We conclude that the S1 working stroke occurs within 5 ms of myosin binding to actin.

The myosin-I used in this study supports actin filament movement

in vitro, although at slower speeds than skeletal-muscle myosin S1 (130K myosin-I¹⁴ (myr-1), $0.04 \mu\text{m s}^{-1}$; BBM-I¹⁵, $0.08 \mu\text{m s}^{-1}$; skeletal S1^{16,17}, $2\text{--}7 \mu\text{m s}^{-1}$ at 37°C). These findings show that the slower myosins have longer working strokes. Differences in *in vitro* sliding velocities must therefore be explained by the much slower biochemical kinetics (ADP release and ATP binding) and longer actomyosin attached lifetimes.

It is thought that the myosin regulatory domains might act as lever arms to amplify motions of the catalytic domains¹⁸. We found that the size of the second step measured for BBM-I (5.5 nm) is similar to the regulatory domain motion induced by ADP binding (deduced from cryoelectron microscopy reconstructions, 6.3 nm)¹⁹. Myr-1 had a similar-sized second step (Fig. 3a), despite its regulatory domain being twice as long as that of BBM-1. This can be explained only if motions of the catalytic domains of these myosins are different (Fig. 3b). The *in vitro* measurements made here are necessarily unphysiological, especially the manner in which the myosins are adsorbed to the nitrocellulose substrate, so it is possible that the *in vivo* movements produced might be larger than the values reported here.

We conclude that two members of the myosin-I family produce a working stroke that occurs in two phases and that the phases are probably linked to different biochemical states of the actomyosin cycle. Further investigation of myosin-II with slower kinetics and other members of the myosin superfamily should show whether the

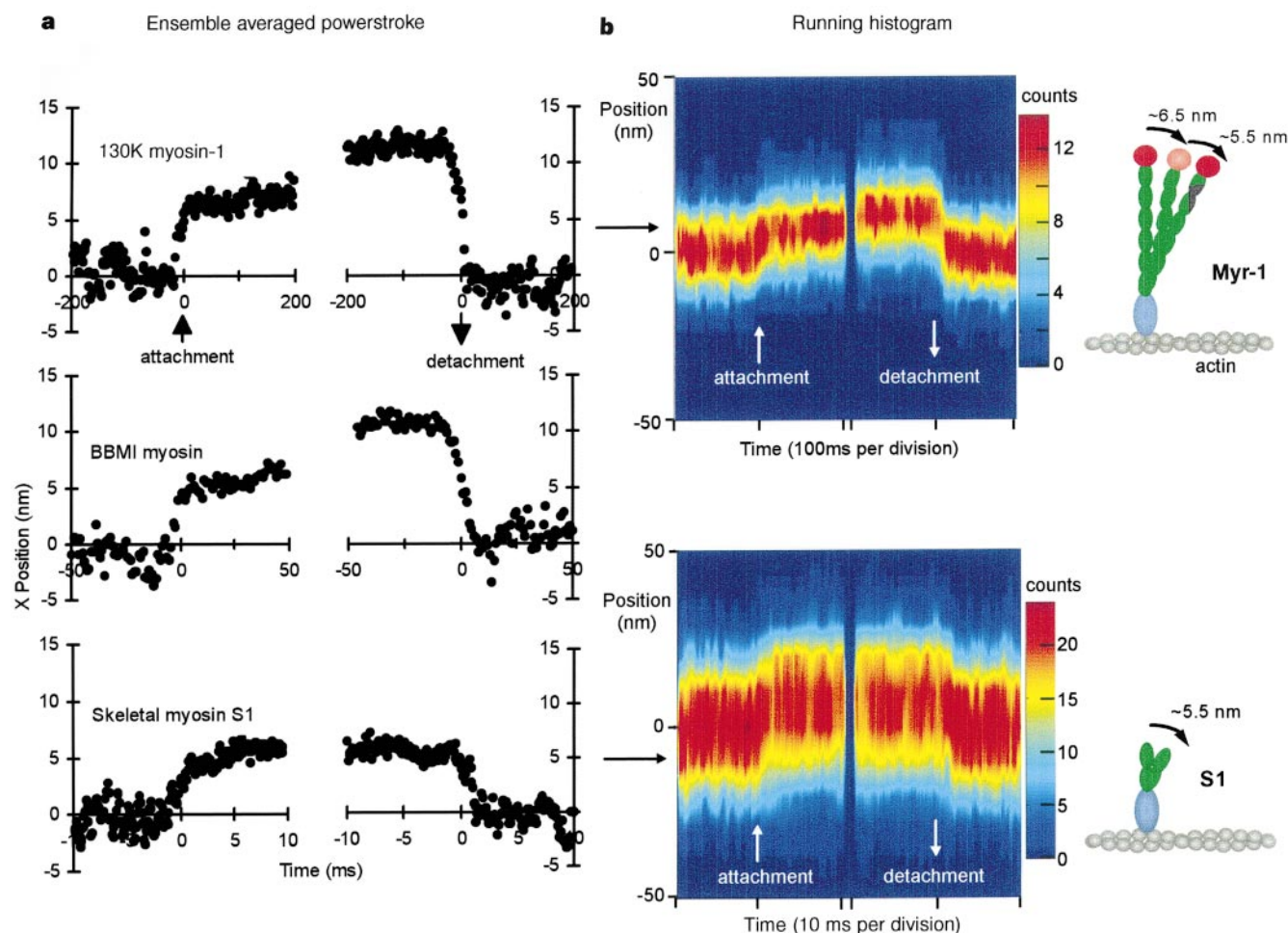


Figure 3 Averaged records of myosin binding to actin. **a**, Left, displacement data from n events were synchronized to the start and end of each interaction (Fig. 2) and the data at each digital sampling point were averaged (myr-1, $n = 63$; BBM-1, $n = 98$; S1, $n = 88$). Running histograms were plotted (**b**) to show how the raw data were distributed. The raw data were synchronized as before and the records superimposed and colour-coded to represent the average number of data points

in each position bin (bin sizes, S1 = 10 nm; myr-1 = 5 nm) at each time slice (3 digital sample intervals). The running histogram obtained for S1 is broader because of the added sinusoidal oscillation (see Methods). The diagrams (right) show how the regulatory domains (containing either 2 or 6 light chains) might act as lever arms to amplify the conformational changes in the catalytic domains.

multistep working stroke found here is a common feature of the mechanism of force production by myosin motors, or whether it is a peculiar feature of the myosin-I used in this study. □

Methods

Protein preparations and solutions. Rat liver myosin-I (130K, the myr-1a gene product) and chicken brush-border myosin-I were isolated by gel filtration, anion and cation exchange chromatography as described^{7,13}. Rabbit skeletal-muscle myosin S1, rhodamine-phalloidin-labelled F-actin and N-ethyl maleimide (NEM)-modified myosin were also prepared by standard methods^{20,21}.

We used an optical tweezers transducer that is built around a Zeiss Axiovert microscope⁶. Experiments were performed within a 'flow-cell' made from a microscope slide and pieces of coverslip. Glass microspheres (1.7 µm) were applied to the coverslip surface as a suspension in 0.1% w/v nitrocellulose/amyl acetate. After drying this was made into a 0.1-mm-deep flow-cell and either BBM-1, myr-1 or skeletal S1 was allowed to bind to the coverslip surface using 0.2, 0.2 and 1 µg ml⁻¹ of protein, respectively, in a buffered salt solution²¹ (containing in mM: 25 KCl, 25 imidazole, 4 MgCl₂, 1 EGTA, pH 7.4, 23 °C). The solution was replaced with one containing rhodamine-phalloidin-labelled actin filaments and 1.1 µm polystyrene beads which were pre-coated with NEM-modified myosin (and the buffered salt solution was supplemented with, in mM: 2 creatine phosphate, 20 dithiothreitol (DTT), 0.01 to 0.1 ATP; and in mg ml⁻¹: 1 creatine phosphokinase, 0.5 BSA, 3 glucose, 0.1 glucose oxidase, 0.02 catalase²²). A single actin filament was captured between two polystyrene beads held suspended in mid-solution in two independent optical tweezers^{23,6} in the vicinity of the stationary glass microsphere. Its interaction with the surface-bound myosin was monitored by casting the image of one of the latex beads onto a 4-quadrant photo-detector. With the actin filament held taut but in the absence of myosin binding, the r.m.s. amplitude of brownian motion is given by $(kT/2\kappa_{\text{trap}})^{0.5} \sim 8.5$ nm r.m.s. (where kT = thermal energy; κ_{trap} = optical tweezer stiffness = 0.028 pN nm⁻¹). When myosin binds to the actin filament the beads are restrained by an additional stiffness (κ_{add}) which reduces the r.m.s. amplitude of their motion to $(kT/(2\kappa_{\text{trap}} + \kappa_{\text{add}}))^{0.5}$. Brownian motion shows a lorentzian power-density distribution with a roll-off frequency $f_c = \kappa/2\pi\beta \sim 430$ Hz (where $\beta = 6\pi\eta r$; η = solution viscosity, r = bead radius = 0.55 µm).

To improve the time resolution with which the onset of attachments could be detected we oscillated the position of one laser tweezer (at a frequency of $f = 1$ kHz and r.m.s. amplitude, $A_0 = 90$ nm) and monitored transmission of this motion to the other bead. The combined effect of viscous damping and series elastic elements⁹ caused the amplitude of the resultant right-hand-side bead movement to be much smaller and to drop dramatically upon myosin binding ($A_r \approx (A_0\kappa_{\text{trap}})/(\kappa_{\text{tot}}^2 + (2\beta 2\pi f)^2)^{0.5}$; where $\kappa_{\text{tot}} = 2\kappa_{\text{trap}} + \kappa_{\text{add}}$ and during myosin attachment κ_{add} increases from zero to about 0.4 pN nm⁻¹). By oscillating the optical tweezer in this way it was possible to identify the start and end of each skeletal muscle myosin attachment from the r.m.s. amplitude of the 1 kHz sine wave with a time resolution of ~ 1 ms.

To ensure that the records we obtained derive from single myosin heads, we first made measurements at high surface densities of myosin and found that the actin filament was translated by several hundred nanometres, often pulling the beads completely out of the optical tweezers, under these conditions the bound lifetime was very non-linear with [ATP]. Then the myosin surface density was adjusted by diluting the myosin-containing solution such that single isolated events with long intervening periods of free bead diffusion were observed. At this surface density of myosin, about half of the stationary beads tested showed no interactions with the actin filament (we could find no active myosin on any region of the bead). Electron micrographs (not shown) showed that both myr-1 and BBM-1 deposited as monomers on nitrocellulose and did not clump or dimerize on the surface. Furthermore, the single extra step observed in the myosin-I records could be readily identified in about 80% of the events. Stiffness of the optical tweezers (κ_{trap}) was calibrated by applying a large amplitude triangular waveform displacement to the microscope stage (stage velocity, v) and by measuring the resulting bead displacement (d) caused by viscous drag force; giving $\kappa_{\text{trap}} = \beta v/d$. The 4-quadrant photodetector was calibrated by moving the trapped bead by a known amount using the laser beam steering devices. The bandwidth of the 4-quadrant photodetector was

5 kHz. Data were sampled at 10 kHz, digitized to 12 bits and stored on computer disk.

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Structural basis for self-association and receptor recognition of human TRAF2

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Tumour necrosis factor (TNF)-receptor-associated factors (TRAFs) form a family of cytoplasmic adapter proteins that mediate signal transduction from many members of the TNF-receptor superfamily and the interleukin-1 receptor¹. They are important in the regulation of cell survival and cell death. The carboxy-terminal region of TRAFs (the TRAF domain) is required for self-association and interaction with receptors. The domain contains a predicted coiled-coil region that is followed by a highly conserved

TRAF-C domain². Here we report the crystal structure of the TRAF domain of human TRAF2, both alone and in complex with a peptide from TNF receptor-2 (TNF-R2). The structures reveal a trimeric self-association of the TRAF domain, which we confirm by studies in solution. The TRAF-C domain forms a new, eight-stranded antiparallel β -sandwich structure. The TNF-R2 peptide binds to a conserved shallow surface depression on one TRAF-C domain and does not contact the other protomers of the trimer. The nature of the interaction indicates that an SXXE motif may be a TRAF2-binding consensus sequence. The trimeric structure of the TRAF domain provides an avidity-based explanation for the dependence of TRAF recruitment on the oligomerization of the receptors by their trimeric extracellular ligands.

To understand the molecular basis of TRAF self-association and receptor interaction, we determined the crystal structures of the TRAF domain of human TRAF2, alone and in complex with a TNF-R2 peptide (Fig. 1). We solved the structure of the peptide-free form at 2.6 Å resolution by the multiple isomorphous replacement method supplemented with six-fold non-crystallographic symmetry averaging. The refined structure at 2.2 Å resolution includes

residues 334–501 for each protomer of the two trimers in the crystal. The structure in complex with the receptor peptide (420'–QVPFSKEEC-428'; numbers marked by a prime symbol denote receptor residues), which has been shown to interact with TRAF2 (refs 2, 3), was determined by molecular replacement and refined at 2.3 Å resolution. It includes residues 311–501 for one trimer and residues 316–501 for the other trimer in the crystal. Two receptor peptides were visible for the first trimer and crystal packing contacts prevented peptide binding to the remaining four protomers, consistent with the expected low affinity of the monomeric receptor peptide. It is unlikely, however, that the observed peptide binding is a crystallographic artefact as the two copies of the peptide make essentially identical contacts with TRAF2.

Each protomer of the TRAF2 trimer contains two domains (Fig. 1). As expected from sequence analysis, the coiled-coil domain (up to residue 347) forms a single α -helix (α_N). Residues in this domain appear to be more flexible and exhibit higher average temperature factors than residues in the other domain. The TRAF-C domain, as identified from sequence analysis (residues 352–501), forms an eight-stranded antiparallel β -sandwich, with strands β_1 ,

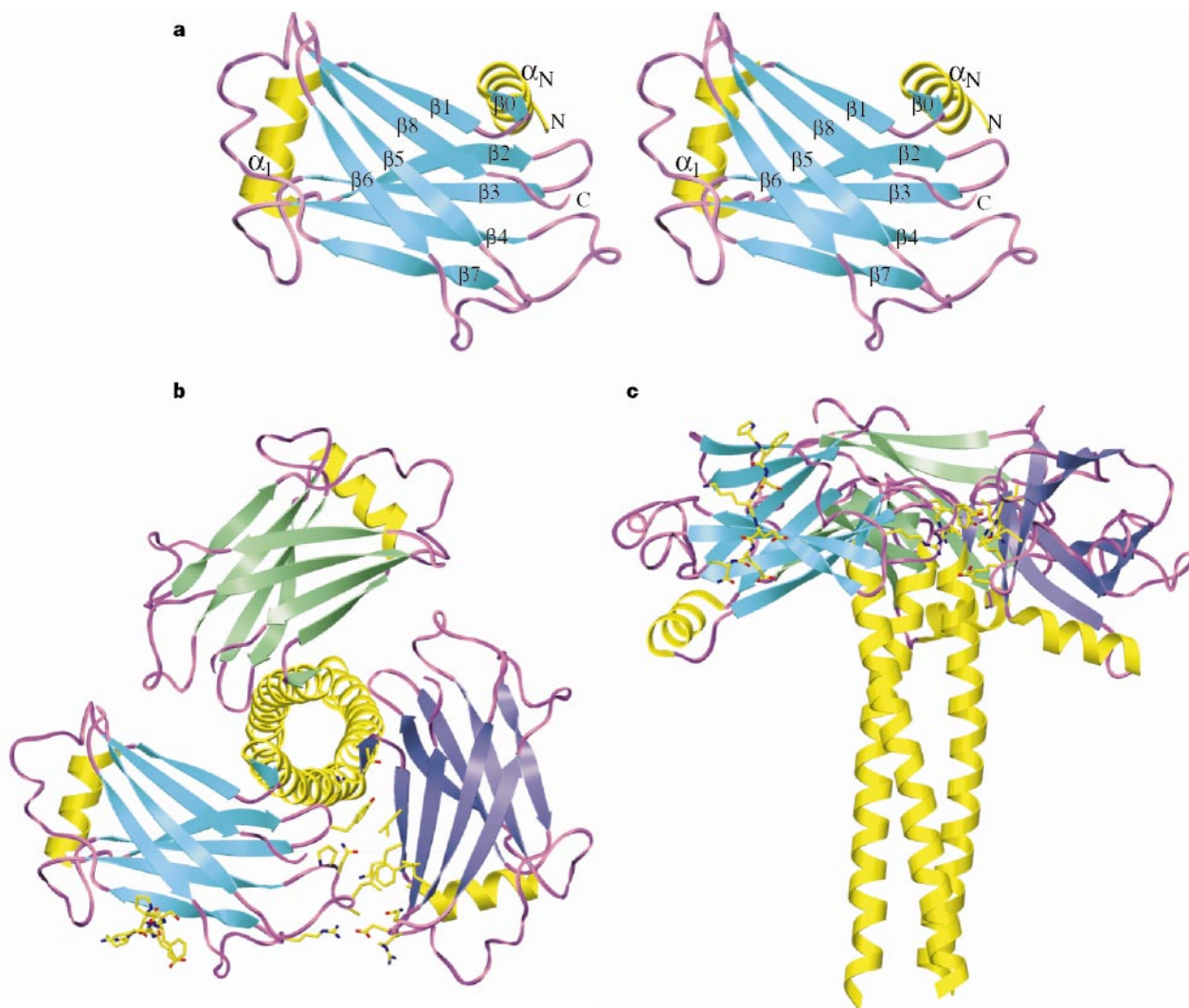


Figure 1 Structure of the TRAF domain alone and in complex with the TNF-R2 peptide. **a**, Stereo ribbon diagram of the TRAF domain of human TRAF2 in the peptide-free structure. The β -strands, α -helices and loops are shown in cyan, yellow and purple, respectively. The loop between β_7 and β_8 is highly flexible and exhibits a different conformation in the peptide-bound structure **b**, Ribbon drawing of the trimeric TRAF domain in complex with TNF-R2 peptide, looking down the three-fold axis. The β -strands in each protomer are shown in cyan,

green and dark blue. The peptide is shown as a stick model for the protomer in cyan. Residues of the TRAF-C domain in the trimer interface (between the protomers shown in cyan and dark blue) are also shown as stick models. The TRAF-C domain of the structure obeys proper three-fold symmetry, whereas the coiled-coil domain shows significant deviations. **c**, As for **b**, except that the three-fold axis is now vertical.

β 8, β 5 and β 6 in one sheet and β 2, β 3, β 4 and β 7 in the other. Strands β 2 and β 7 each contain a β -bulge, which seems to have important structural and biological functions (see below). A three-turn helix is present in the crossover connection between strands β 1 and β 2. Preceding the β -sandwich, residues 348–350 form a parallel β -structure (β 0) with strand β 2, immediately after the β -bulge in this strand. The side chains of residues in β 0 also partly cover one edge of the β -sandwich. Therefore, structural analysis suggests that residue 348 should be the start of the TRAF-C domain, even though residues 348–351 exhibit sequence variability. On the basis of visual inspection of the SCOP structure database⁴ and an automatic structural-similarity search using the Dali program⁵, the TRAF-C domain appears to represent a new fold for an eight-stranded anti-parallel β -sandwich. However, the topology observed for this domain may be reached by circular permutations of the β -strands in copper–zinc superoxide dismutase (Protein Data Bank entry 2SOD)⁶ and the C2 domain from synaptotagmin I (PDB entry Isry)⁷.

The TRAF-domain trimer has the shape of a mushroom, with the TRAF-C domain as the cap and the coiled-coil domain as the stalk (Fig. 1). The diameter of the mushroom cap ranges between 50 and 80 Å and a stalk containing five heptad repeats is 50 Å in length (residues 311–347). The parallel coiled-coil structure is formed by the hydrophobic residues at positions a and d of the heptad repeats (Fig. 2) that are characteristic of coiled-coil structures⁸. The trimer interface of the TRAF-C domain is formed by packing one end of the β -sandwich (the β 2– β 3, β 4– β 5 and β 6– β 7 connections) against an edge and a face of the β -sandwich (β 0, β 1 and β 8 strands, β 5– β 6 and β 7– β 8 connections) of the neighbouring protomer. Most residues that participate in the formation of this interface are rather hydrophobic (such as I355, Y386, A420, L421

Table 1 Receptor–TRAF2 interactions

TNF-R2	S	Area (Å ²)	VDW contacts	Potential hydrogen bonds
P422'	0.71	44	2	
F423'	0.69	73	6	
S424'	0.01	101	14	O γ : 467O γ
K425'	0.73	67	10	N: 468O; O: 468N
E426'	0.50	91	8	
E427'	0.13	139	18	N: 466O; O ϵ 1; 395O η ; O ϵ 1; 393N η 1; O ϵ 2: 393N η 1
C428'	0.66	58	5	N: 399O δ 2

S, fraction solvent accessibility of the receptor side chains; area, total surface area buried upon binding for each receptor residue; VDW contacts, number of atoms in TRAF2 that make van der Waals contacts with the receptor residue.

and F491). Hydrophilic interactions are also observed at this trimer interface, involving the side chains of K357, R385, R458 and D487. The amino-acid residues contributing to trimerization of the TRAF domain are conserved among the TRAF-family members (Fig. 2). This sequence conservation indicates that other TRAF molecules may be able to form similar homotrimers as well. Formation of heterotrimers, as has been shown for TRAF1 and TRAF2 (ref. 2), may also be possible.

The trimeric self-association of TRAF2 was confirmed by solution studies. Size characterization of a TRAF2 protein containing residues 310–501 (relative molecular mass 22,600 (M_r 22.6K)) by equilibrium ultracentrifugation showed that it is a homogeneous species of M_r 63.3K. Chemical crosslinking with glutaraldehyde produced a single band on SDS–polyacrylamide gels of approximate 65K. Similar studies in conjunction with gel-filtration analyses showed that the TRAF-domain molecule containing residues 327–501 was also trimeric. However, the TRAF domain containing residues 342–501 was monomeric. These observations indicate that the stability of the trimer may be dependent on both the coiled-coil domain and the TRAF-C domain, in agreement with earlier biological studies⁹. Structurally, each TRAF-C domain alone buries a surface area of roughly 640 Å² upon trimerization; this area is rather small compared with that produced by other stable protein–protein interactions¹⁰. The results of the solution studies indicate that a minimum of roughly three heptad repeats (residues 327–347), which produces an additional surface burial of 420 Å², may be required for trimer formation. The coiled-coil domain of TRAF4 contains only three heptad repeats, the fewest of all the TRAFs. In comparison, the coiled-coil domain of TRAF2 appears to contain up to 14 heptad repeats.

Each TNF-R2 peptide is bound symmetrically to a shallow surface depression on the side of the mushroom-shaped trimer, extending from the top to the bottom rim of the mushroom cap (Figs 1, 3). The peptide contacts only one TRAF domain, with no contacts to the other two molecules of the trimer. This mode of interaction is distinct from the interaction between TNF and the extracellular domain of its receptor; each receptor binds near the interface between neighbouring protomers in the TNF trimer¹¹. We observed no significant conformational changes near the receptor-binding sites or in the relative orientations of the protomers in the trimer upon receptor binding, although there are conformational differences in several flexible regions of the TRAF domain.

The receptor peptide assumes a nearly extended main-chain conformation in the complex (Fig. 3). The receptor-binding site in TRAF2 is formed by the exposed faces of strand β 6 in the first sheet and strands β 7, β 4 and β 3 in the second sheet. A portion of the peptide chain (K425'–E427') runs antiparallel and adjacent to the latter half of strand β 7 (residues 466–468), immediately after the β -bulge in this strand. This leads to three main-chain hydrogen bonds between the peptide and TRAF2, extending the four-stranded second β -sheet by one strand. In addition, the main-chain amide group of C428' is within hydrogen-bonding distance of the carboxylate group of D399 in TRAF2. The formation of a β -sheet has been frequently observed in protein–peptide interactions, for example in the interaction between substrates and certain serine

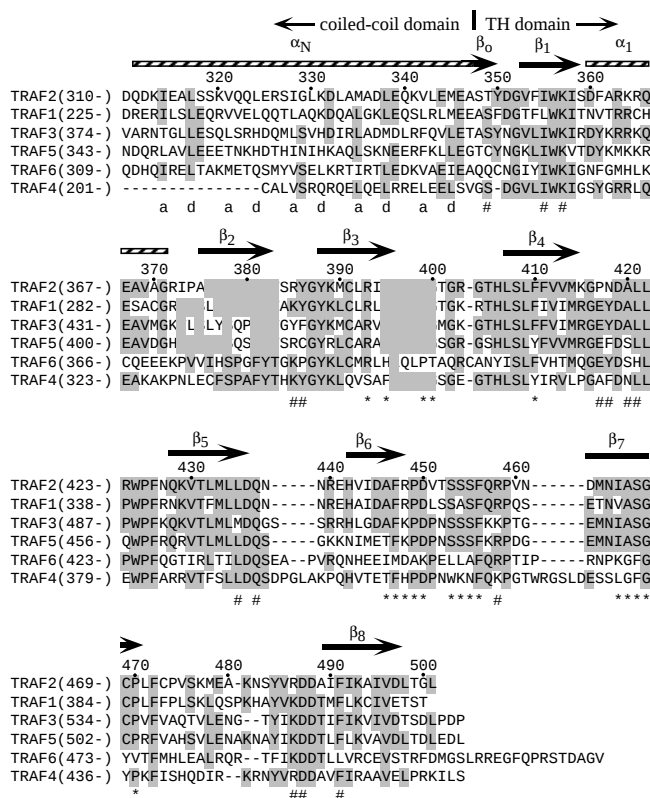


Figure 2 Structure-based sequence alignment of TRAF-family members. Secondary structures, the a and d positions of the coiled-coil domain, residues in the TRAF-C domain involved in trimerization (hash symbols), and residues interacting with the receptor peptide (asterisks) are mapped onto the TRAF2 sequence. Residues conserved among at least three TRAF family members are shaded.

proteases¹² and between peptides and the PTB and PDZ domains¹³.

Residues P422'–C428' of the receptor peptide show interactions with the TRAF2 protein (Table 1, Fig. 3), burying a surface area of 500 Å² upon binding. Residues Q420'–V421' are ordered in only one copy of the peptide, where the peptide is stabilized by crystal packing. It is likely that residues preceding P422' do not contribute to the binding. The peptide makes mostly van der Waals contacts with TRAF2, of which ~50% are polar–nonpolar contacts, 35% are nonpolar–nonpolar contacts and 15% are polar–polar contacts. Residues between F423' and C428' appear to make significant contributions to the overall binding energy, as judged by the surface area buried and the number of TRAF2 atoms in contact with these residues (Table 1). It remains to be seen whether residues following C428', which are not included in the receptor peptide used here, can also contribute to binding. Most of the receptor residues are bound to shallow pockets on the surface of the TRAF-C domain and are largely exposed to solvent, consistent with the expected low affinity of the interaction.

Residues S424' and E427' of the receptor peptide appear to be recognized specifically by TRAF2. The side chain of S424' binds to a small pocket on the surface of the protein and becomes completely buried. The hydroxyl group of S424' is within hydrogen-bonding distance of the hydroxyl group of S467 (in strand β7) of TRAF2. The side-chain carboxylate group of E427' forms an ion pair with R393 (β3), and is also hydrogen-bonded to the side-chain hydroxyl of Y395 (β3). The aliphatic portion of the E427' side chain is mainly buried, leaving 13% surface exposure for the entire side chain. It therefore appears that these two receptor residues are the major determinants of binding specificity, giving rise to an SXXE motif, where X may be one of many amino-acid residues, preferably one with a relatively large side chain. Further support for the importance of this binding motif comes from the observation that the main-chain hydrogen-bonding interactions between this peptide and TRAF2 are also mediated through these residues. They are then likely to serve as anchoring points during binding.

A homologous sequence from the receptor CD30 (residues 576–MLSVEEE–582), a member of the TNF-receptor superfamily, interacts with TRAF2 (ref. 3). The conservation of the SXXE motif in the CD30 receptor indicates that its binding to TRAF2 is likely to be the same as observed here for the TNF-R2 peptide. In agreement with our structural observations, single-point alanine mutagenesis of the CD30 peptide showed that residues L577–E581 each contributed to the overall binding energy, as assessed by co-precipitation experiments³. Further mutagenesis and quantitative binding studies are needed to determine more precisely the energetic contribution and the tolerance to mutations for each residue of the receptor peptide.

The three residues of TRAF2 (R393, Y395 and S467) that recognize the key residues in the SXXE motif of the receptor peptide are strictly conserved among TRAFs 1, 2, 3 and 5 (Fig. 2). In TRAF4, the three residues are changed to serine, phenylalanine and phenylalanine, respectively. TRAF4 appears to be localized in the nucleus¹⁴ and has not yet been shown to interact with any receptors. In TRAF6, R393 is conserved, whereas Y395 and S467 are changed to histidine and phenylalanine, respectively. The S467F change introduces a bulky phenyl ring on the surface, which may not allow the receptor peptides to bind in the same way as observed here. It is not clear from our structure why the TNF-R2 peptide binds only to TRAF1 and TRAF2, and not to TRAF3, as shown by co-precipitation experiments³. It is likely that this selectivity is caused indirectly by sequence variations outside the binding site. One such possibility is the V451P variation in TRAF3 (and in TRAFs 4–6; Fig. 2), which might change the local helical conformation observed in this structure and therefore alter the positioning of several adjacent residues involved in the peptide interaction (Fig. 3). In a similar way, adenylyl cyclase is activated by the G protein G_{sα} rather than G_{iα}, as revealed by crystallographic studies¹⁵.

TRAF proteins interact with several other linear sequences from

the intracellular domains of members of the TNF-receptor superfamily¹. For example, a PXQX(T/S) TRAF-binding motif (where X may represent any amino acid) has been identified in CD30, CD40, CD27 and LMP-1 (ref. 1). Stretches of conserved acidic residues have also been shown to mediate the binding of the receptors 4-1BB and Ox40 to TRAF molecules¹⁶. In addition, TRAF6 may bind to CD40 and the p75 neurotrophin receptor through sequences completely different from those described above^{17–19}. Whether these diverse motifs bind to the SXXE site identified here, perhaps also exploiting β-edge hydrogen bonding, or to a different TRAF site, remains to be determined by structural studies.

TRAF molecules are important mediators of signalling through

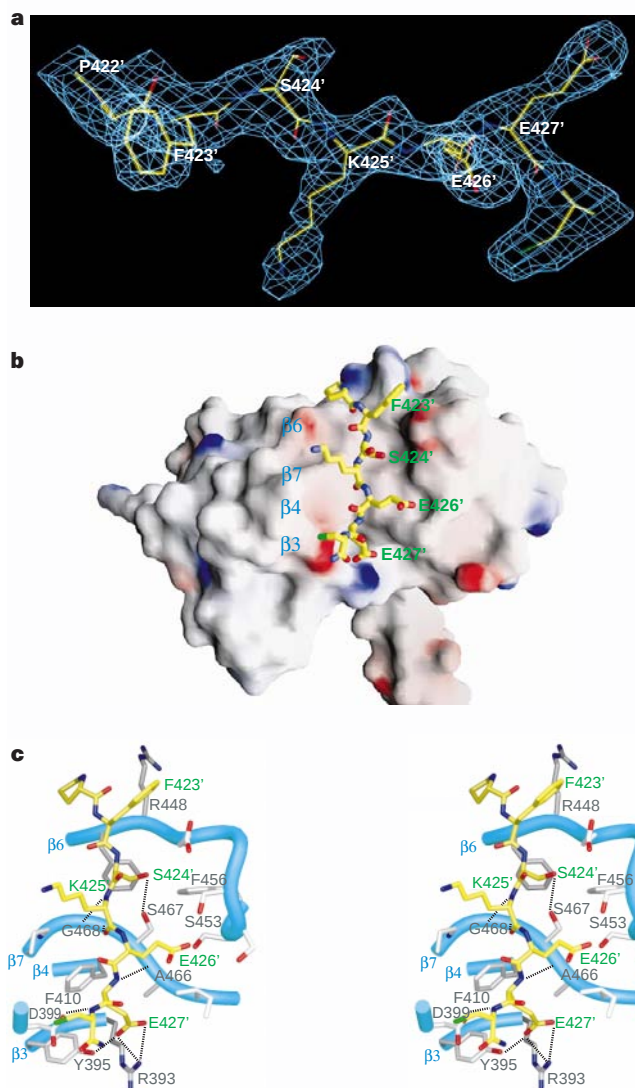


Figure 3 Detailed interaction between TRAF2 and the TNF-R2 peptide. **a**, Simulated annealing omit difference map for the TNF-R2 peptide calculated with reflections between 20.0 and 2.3 Å resolution and contoured at 2.0σ. The peptide model is superimposed. **b**, Molecular surface of a TRAF2 protomer, showing the bound TNF-R2 peptide as a stick model; the three-fold axis is in the vertical orientation. Surface colour coding is according to electrostatic surface potential, scaled from –30 to +30 kTε⁻¹, with blue for positive and red for negative. Selected residues in the receptor peptide and the underlying secondary-structural elements of TRAF2 at the binding site are labelled. **c**, Stereo view of the detailed interaction between the TNF-R2 peptide (carbon atoms shown in yellow) and the TRAF2 protomer (carbon atoms shown in grey). The main chain of the TRAF2 structure is shown in cyan as backbone worms. Selected residues in the peptide (primed numbers in green) and the protein (in grey) are labelled. Hydrogen bonds and a salt bridge are shown as black dotted lines.

Table 2 Phase determination and structure refinement

Phase determination		Protein construct	Resolution	R_{merge}	Completeness	R_{scale}	Phasing power	
Crystal								
Native		327–501	40.0–2.5 Å	6.2% (26.0%)	99.0% (99.1%)			
Thimerosal (1)		327–501	40.0–2.6 Å	9.2% (31.6%)	94.9% (93.5%)	33.0%	1.9	
Thimerosal (2)		327–501	40.0–2.7 Å	71% (23.0%)	94.6% (92.3%)	32.8%	1.9	
Structure refinement		Peptide	Resolution	R_{merge}	Completeness	Solvent atoms	Average B	R
Protein construct								R_{free}
327–501		No	20.0–2.2 Å	4.3% (10.7%)	99.5% (98.5%)	743	22.2 Å ²	20.9%
310–501		Yes	20.0–2.3 Å	4.8% (27.9%)	99.0% (97.9%)	910	47.1 Å ²	23.4%

Statistics for the last resolution shell are shown in parentheses. R_{scale} is the R -factor between native and derivative amplitudes.

members of the TNF-receptor superfamily. The extracellular events of this signalling process appear to involve the trimerization of the extracellular domains of the receptor molecules by the inherently trimeric ligands of the TNF superfamily. Ligand-induced receptor trimerization has been demonstrated by the co-crystal structure in the interaction between TNF- β and TNF-R1 (ref. 11). The significant sequence homology shared by the superfamily indicates the conservation of this extracellular interaction. The trimerization of the extracellular domains can bring the intracellular domains of the three receptor molecules into proximity, which may then be recognized optimally by the trimeric TRAF2 molecules. The avidity contribution of this oligomeric interaction is expected to increase the affinity exponentially, providing an explanation for the ligand-induced recruitment of TRAF proteins^{1,20}. In addition to direct interactions between receptors and TRAFs, TNF-R1 recruits TRAF2 indirectly through the adapter protein TRADD²¹. It remains to be demonstrated whether TRADD is also trimeric and therefore relays the avidity component of the interaction from the receptor to TRAF2.

The downstream events of TRAF-mediated signal transduction are thought to involve the activation of MAP kinases and, ultimately, transcription factors of the Rel and AP-1 family¹. This signalling process requires the amino-terminal RING and zinc-finger motifs of the TRAF molecules, although the detailed mechanisms of the process are largely unknown. Our structures show that the TRAF domain of TRAF2 may be a constitutive trimer and binds the receptor peptide without significant conformational changes. This argues against receptor-induced TRAF oligomerization or conformational change as the initiator of downstream events. However, such possibilities cannot be excluded on the basis of our data, as full-length TRAF proteins may exist in a different conformation in the unbound state. Alternatively, the recruitment of TRAFs to the cell membrane alone by the receptors might be the determining factor in the signal transduction. □

Methods

Protein preparation and crystallization. The complementary DNA encoding human TRAF2 was obtained from the library of expressed sequence tags. Two constructs of the TRAF domain of TRAF2 (residues 315–501 and residues 310–501) containing carboxy-terminal polyhistidine tags were generated using the pET24d vector (Novagen) and overexpressed in *E. coli* by induction at 20 °C. The recombinant proteins were purified by nickel-affinity chromatography (Qiagen) followed by gel filtration (Superose 12, Amersham Pharmacia). The protein containing residues 315–501 was treated with trypsin at 4 °C for 2 h at a 1:1,000 molar ratio, which removed residues 315–326. The digested protein was further purified and then crystallized at 20 °C from a solution containing 11% PEG4000 and 0.1 M MES, pH 6.0. The crystals belong to space group C2 with cell dimensions of $a = 134.9$ Å, $b = 85.0$ Å, $c = 124.1$ Å and $\beta = 118.6^\circ$. The protein containing residues 310–501 was crystallized from 12% PEG4000 0.2 M ammonium acetate, and 0.1 M citrate, pH 5.6, in the presence of an equal molar concentration (1 mM) of the peptide from TNF-R2 (residues 420'–QVPFSKEEC–428'). The peptide was chemically synthesized with N-terminal acetylation and C-terminal amidation to mimic the intact protein. The estimated K_d of the interaction (0.1–1.0 mM; preliminary results) indicates that the binding sites may not be fully saturated under these crystallization conditions. Although similar crystals can be obtained in the absence of the peptide, higher concentrations of the peptide prevented crystal growth,

indicating competition with crystal packing. The crystals belong to space group $P2_1$ with cell dimensions of $a = 83.0$ Å, $b = 84.4$ Å, $c = 100.7$ Å and $\beta = 108.7^\circ$. Both crystal forms contain two trimers of the TRAF2 protein per crystallographic asymmetric unit.

Structure determination and analysis. All X-ray diffraction data (Table 2) were collected on cryoprotected crystals and processed with the HKL package²². Native and derivative data for the peptide-free crystals were measured on an R-axis IV imaging plate system mounted on a Rigaku RU300 rotating anode X-ray generator. Heavy-atom positions were located from the isomorphous differences by direct methods using SHELX²³; some of these positions were also found by Patterson interpretation using PATSOL²⁴. MIR phases were calculated for reflections up to 2.6 Å resolution with the program PHASES²⁵. After solvent-flattening, the phases were further improved by six-fold non-crystallographic symmetry (NCS) averaging with a locally written program (L.T., unpublished results). Sequence fitting and model building were done with program O (ref. 26). A 2.2 Å data set collected using the CCD detector at the CHESS F2 beamline was used for the crystallographic refinement, with the program CNS²⁷. NCS restraints were used throughout the refinement. Diffraction data of the peptide complex were collected at the X4A beamline of NSLS. The structure was determined by molecular-replacement calculations with the program REPLACE²⁸ using the trimer atomic model from the peptide-free structure and refined at 2.3 Å resolution using program CNS²⁷. An $|F_o| - |F_c|$ difference map clearly showed the electron densities for the bound peptides and for the extra N-terminal residues. The coiled-coil region of the protein deviates from non-crystallographic symmetry and NCS restraints were applied to the TRAF-C domain alone. Molecular surface representations and stick models were produced with the program GRASP²⁹ and ribbon diagrams were generated using the program SETOR³⁰.

Characterization of oligomerization in solution. Equilibrium sedimentation was done at 20 °C on protein samples at 25 μ M concentration using an XL-A Optima analytical ultracentrifuge (Beckman) running at 11,000 r.p.m. Data were fit to a single species model. The apparent relative molecular mass was calculated by assuming a partial specific volume of 0.74 ml g⁻¹ and a solvent density of 1.045 g ml⁻¹. Chemical crosslinking was done at room temperature with 5 μ M protein samples in the presence of an 8,000-fold molar excess of glutaraldehyde. The reaction mixtures were quenched with excess glycine after 40-min incubations, concentrated using Microcons (Amicon) and separated by SDS-PAGE.

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Correspondence and requests for materials should be addressed to H.W. (e-mail: haoxu@mail.med.cornell.edu). Atomic coordinates have been deposited in the Brookhaven Protein Data Bank under accession numbers 1ca4 and 1ca9.

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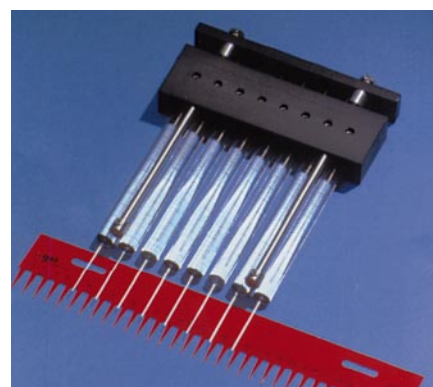
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channel 9 mm spacing syringes such as the Hamilton. These vinyl and Mylar sharkstooth combs, in lengths of 15 cm or 30 cm. and thicknesses of 0.4 mm, 0.35 mm or 0.25 mm, are compatible with most popular gel systems. Select from numbered wells of 33, 50, 67, 101 or 135. Choice of 1.0 cm or 1.5 cm tooth depth and point to point spacing of 2.25 mm, 3.0 mm or 4.5 mm. Full line of compatible spacers also available.

Reader Service No. 115

Stainless-steel vessels

From LabPlant

www.labplant.com

Containers that can take the pressure

The new range of stainless steel reaction vessels from LabPlant covers 2 to 100 litres capacity and features many variations and accessories custom-build equipment to customer's requirements. Designed to operate at up to 7 bar at 200°C the standard range includes a jacket for the vessel which can be temperature controlled by oil circulation using high-performance thermostats or by steam. The lids have a stirrer gland assembly, an inspection glass and light, pressure gauge, pressure relief valve and standard or specified openings for connection to vacuum or pressure or for filling or condensing.

Reader Service No. 116

ADVERTISEMENTS

STERNBERGER MONOCLONAL (SMI) ANTIBODIES FROM AFFINITI

The SMI series of antibodies to neurofilaments, tau, myelin basic protein, blood brain barrier (EBA), SNAP-25, and tubulins is available from AFFINITI.

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For data sheets or more information on AFFINITI's extensive range of products for neuroscience research please visit our website at:

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READER ENQUIRY NO. 15

The Mouse KitSM for Gene Targeting in ES Cells

- ✓ Complete with embryonic stem (ES) cells, feeder cells and essential reagents (including FBS) for engineering one knockout mouse strain
- ✓ Low-passage AB2.2-Prime ES Cells[®] (129/SvEv)
- ✓ Uniform, high-quality ESQ feeder cells (mitotically inactivated STO cells) engineered for *neo* and *puro* resistance
- ✓ Prequalification of ESQ feeder cells and reagents for use with AB2.2-Prime ES Cells

*Manufactured by Lexicon Genetics Incorporated



Tel: (619) 535-5400 or (800) 424-5444

www.stratagene.com

READER ENQUIRY NO. 6

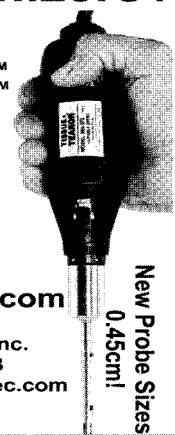
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New Probe Sizes
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READER ENQUIRY NO. 54

Centrifugation

Sigma 3K30

From Philip Harris Scientific

www.philipharris.co.uk/scientific

High-speed centrifuge with one-touch controller

The Sigma 3K30 has a top speed of 30,000 r.p.m. and can generate 64,000g with fixed angle and 5,000g with swing-out rotors. A selection of 13 hermetically sealed rotors is available, one swing-out and 12 fixed-angle up to a capacity of 6 × 85 ml. All rotors can be pre-cooled at stand-still. The 3K30 is microprocessor controlled via a one-touch controller. Time, speed, rotor, gravitational field and temperature can all be preselected and displayed on a large LCU screen.

Reader Service No. 117

Biofuge Pico

From Kendro Laboratory Products

www.kendro.com

An ultra-compact microcentrifuge

Biofuge Pico is capable of spinning 24 tubes, either 1.5 or 2 ml, at up to 13,000 r.p.m. (16,060g). The centrifuge is compact, at 9 × 13 inches, and features a load-relief lid and an ergonomically designed lid grip for easy access to the rotor. The same running gear is available in a refrigerated model, Biofuge Fresco. Rotors for the Pico and Fresco have Porton Down approved biocontainment lids for use with infectious or hazardous samples. Hermetically sealing screw-on lids complement the rotors' transparent snap-on lids, which are used for routine spins.

Reader Service No. 118

Spectroscopy

BioPhotometer

From Eppendorf

www.eppendorf.com

For the rapid analysis of nucleic acids, proteins and bacterial cell density

Built to be portable, the BioPhotometer takes 2 seconds to perform a measurement. A set of five programmed methods covers common lab requirements: quantifying ds/ssDNA, RNA; purity check with 260/280 and 260/230 ratios; optional background correction at 320 nm for all UV methods; protein determination by methods including Lowry/Lowry micro, Bradford/Bradford micro and BCA/BCA micro; and bacterial cell density at 595 nm.

Reader Service No. 119

HP 8453E

From Hewlett Packard

www.hp.com

An interactive, online demonstration of a new spectrophotometry system

The demo is accessible through the Web site of HP's Chemical Analysis Group, located at http://chem.external.hp.com/cag/products/universo_welcome.html. The demo gives users the opportunity to interactively set up and make a measurement through the HP 8453E's handheld controller. In addition, users can display the measured spectrum, change wavelength settings and get a simulated printout.

Reader Service No. 120

Cleaning up

LabSolutions

From Labconco

www.labconco.com

Proliferating powders

LabSolutions Powder Detergent is now joined by two variants: LabSolutions Liquid Detergent, and the New LabSolutions Acid Rinse. The liquid detergent is a low- non-corrosive formulathat won't degrade stainless steel, glass or plastic and is phosphate-



Hot and cold running centrifuges.

free. LabSolutions Neutralising Acid Rinse alters the pH of the wash water, reducing alkaline detergent residue. It eliminates mineral deposits, accumulation of salts, and glassware-clouding lime scale. On Labconco washers, a 34-oz. bottle fills the neutralizing solution reservoir approximately 6 times, for approximately 255 rinses. Labconco also offers LabSolutions Powdered Detergent. The non-foaming powder removes most lab contaminants, from grease and agar to blood and protein digestates.

Reader Service No. 121

Counts Away

From Molecular BioProducts

www.mbpinc.com

A decontaminant to handle radioactive contamination

Counts Away joins RNase AWAY and DNA AWAY in the Molecular BioProducts catalogue. Counts Away is used to decontaminate hard surfaces, by simply soaking the affected surface and then rinsing to remove radioactivity. Counts Away is concentrated to make up to 10 litres of solution but dilution can be altered for tougher jobs.

Reader Service No. 122

Alconox

From Bel-Art Products

www.bel-art.com

A new cleaner — try it for free

Alconox Cleanware is a plastic labware cleaner formulated for Bel-Art Products of Pequannock, New Jersey. It removes residue from plastic labware without scratching, hazing, crazing or stress cracking, even on polycarbonate. It can be used for glassware, ceramics and metals as well. Concentrated for convenient storage, it is formulated at a dilution rate of 1:100 parts and can be used in hot or cold water. Cleanware Plastic Labware Cleaner is biodegradable, has a neutral range pH and is supplied in a one gallon (3.8 litre) plastic bottle. For additional information and a free sample of Cleanware call 1-800-4BELART or visit the website.

Reader Service No. 123

These notes are compiled in the Nature office from information provided by the manufacturers. For more details, fill in the reader service card.



imMedia™ — Fast Food for E. coli

imMedia™ is pre-mixed, presterilized *E. coli* growth medium that can save you hours. With imMedia™ you can prepare medium immediately because there's:

- No mixing of media components
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- No adding antibiotics, IPTG, or X-gal

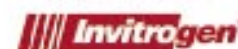
Simply add water and microwave for 3.5 minutes and you are ready to go. When you need media in a hurry, think imMedia™ from Invitrogen. Fast Food for *E. coli*.



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READER ENQUIRY NO. 12